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Elucidating the signal cascades induced by progestins that mediate sperm hypermotility in Atlantic croaker (*Micropogonias undulatus*) and southern flounder (*Paralichthys lethostigma*)

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Dedication

"It is sometimes a mistake to climb, it is always a mistake never even to make the attempt." Dream, *Sandman*: "Fear of Falling"

To Mom, Dad, and Xue. Thank you for believing in my crazy. To Pi. Thank you for being my li'l mammal. I owe you all my success, sanity, and style. In no particular order.

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Elucidating the signal cascades induced by progestins that mediate sperm hypermotility in Atlantic croaker (*Micropogonias undulatus*) and southern flounder (*Paralichthys lethostigma*)

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The University of Texas at Austin, 2013

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The overall goal of this research was to verify the involvement of membrane progestin receptor alpha (mPRα) in mediating progestin-stimulated sperm hypermotility in the Atlantic croaker and southern flounder. Sperm motility in Atlantic croaker and southern flounder were tested with both the endogenous progestin, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) or the selective mPRα agonist, 10-ethenyl-19-norprogesterone (Org OD 02-0). In croaker, the Pi3k/Akt/Pde and ErbB2/Mapk intracellular signaling pathways were examined. The role of mPRα in mediating sperm hypermotility and fertility in southern flounder was also studied. The effects of seasonal hypoxia on sperm motility in croaker were investigated in a field study in the northern Gulf of Mexico in the fall of 2010. Finally, the effects of acidified activator solution (simulating ocean acidification) were studied in the laboratory.

In vitro, Org OD 02-0 mimicked the stimulatory actions of 20β-S in inducing sperm hypermotility and intracellular signaling cascades in croaker and flounder sperm, indicating that mPRα is the mediator of progestin signaling in the sperm of these species. In croaker sperm, both the Pi3k/Akt/Pde and ErbB2/Mapk intracellular signaling pathways were shown to be important mediators of progestin-induced sperm hypermotility, suggesting novel functions of G_{olf} βγ-subunits in teleost sperm. In flounder sperm, mPRα was shown to be important in mediating sperm hypermotility as only high motility sperm with high expression of mPRα were responsive to progestin stimulation, resulting in higher fertilization success compared to low motility sperm. A single LHRHa injection resulted in increased sperm motility and fertility, associated with an increase in mPRα expression in the sperm plasma membrane. The results also suggest that the mPRα/Acy/cAMP pathway first described in croaker sperm is present in flounder sperm. Field studies of male Atlantic croaker exposed to chronic seasonal hypoxia showed that hypoxia exposure resulted in smaller gonads, lower spermatogenesis, reduced testicular mPRα expression, and in some sites, reduced sperm motility. Studies with croaker sperm using acidified activator solution to simulate ocean acidification indicated that croaker sperm were sensitive to environmental insult. Furthermore, the results suggested that the progestin signaling mechanism is more sensitive to changes in ocean pH levels than the mechanism that controls sperm motility.

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Chapter 1: Introduction

Reports of nongenomic actions of steroids date back to as early as the 1960s (Szego & Davis, 1967). Unlike classical mechanisms involving transcription of new mRNA and translation processes, which are generally slow (Thomas, 2008), these responses to steroid sex hormones are often rapid and mediated through second messenger cascades (Wehling and Lösel, 2006). These rapid effects of steroid hormones are initiated at, or near, the cell surface, and are mediated by binding of steroid hormones to specific receptors at the plasma membrane of the target cells (Thomas, 2008). Rapid actions of progestins on sperm physiology have been described in a variety of vertebrate species including the induction of hypermotility in teleost fish (Thomas, 2003; Tubbs and Thomas, 2009; Tubbs et al., 2011) and mammalian sperm (Uhler et al., 1992; Park et al., 2011). While the importance of rapid nongenomic progestin actions on sperm physiology is widely recognized, the mechanisms through which the steroids mediate these effects remain largely elusive (Thomas et al., 2007; Park et al., 2011).

A novel cDNA that bears the characteristics of a membrane progestin receptor (mPR) was recently characterized in spotted seatrout ovaries (Zhu et al., 2003). The encoded protein belongs to the progestin and adipoQ receptor (PAQR) family and is named membrane progestin receptor alpha (mPRα, also known as Paqr7b) (Thomas et al., 2007), and mediates the effects of the maturation-inducing steroid (MIS) in this species through a nongenomic mechanism (Zhu et al., 2003). The same receptor has been characterized in the sperm of three teleost species: the spotted seatrout (Thomas et al.,

1997), the Atlantic croaker (Thomas et al., 2005), and the southern flounder (Tubbs et al., 2011). Progestin stimulated sperm hypermotility in these teleost species have been suggested to be mediated by mPRα (Thomas et al., 1997; Tubbs and Thomas, 2009; Tubbs et al., 2011). Tubbs and Thomas (2009) showed that mPRα is coupled to a stimulatory olfactory G protein (G_{olf}), and the activation of the α-subunit of the G_{olf} protein causes increases intracellular cyclic adenosine monophosphate (cAMP) levels when activated by progestins. Inhibition of membrane-bound adenylyl cyclase (Acy), which catalyzes the formation of cAMP, has been shown to abrogate progestin-induced sperm hypermotility in both the Atlantic croaker (Tubbs and Thomas, 2009) and southern flounder (Tubbs et al., 2011). Thus, progestin mediated sperm hypermotility in teleosts is suggested to signal through the G_{olf} α-subunit, activating the Acy/cAMP pathway.

While there is clear evidence for the involvement of the α-subunit of the G_{olf} proteins in progestin induction of sperm hypermotility in teleosts, comparable information on the possible role of the G protein βγ-subunit in mediating this sperm function is lacking. Some of the signaling cascades commonly associated with the βγ-subunit signaling pathway are the PI3K/AKT (Stephens et al., 1997; Djordjevic and Driscoll, 2002) and epidermal growth factor receptor (EGFR)/ mitogen-activated protein kinase (MAPK) pathways (Filardo et al., 2000). Progestins acting at the cell surface have been proposed to modulate levels of cAMP in Atlantic croaker oocytes via the Pi3k/Akt signal transduction pathway (Pace and Thomas, 2005). Progestin activation of the Pi3k/Akt pathway in Atlantic croaker oocytes was suggested to activate phosphodiesterases (Pde) that in turn breaks down cAMP (Pace and Thomas, 2005). The

PI3K/AKT pathway has been implicated in mediating male fertility in various mammals; the PI3K/AKT pathway has been reported to regulate sperm viability in boar sperm (Aparicio et al., 2007) and the pathway is suggested to regulate sperm motility in hamsters (NagDas et al., 2002) and chicken (Ashizawa et al, 2008). It has also been suggested to be involved in the mediation of the acrosome reaction in human sperm (Fisher et al., 1998). Given the prevalence and importance of the PI3K/AKT pathway in vertebrate sperm, the possible role of the pathway in mediating progestin-stimulated sperm hypermotility in teleost sperm was explored in this study, using the Atlantic croaker as a model species.

There are no reports on the possible role of EGFR and MAPKs in teleost sperm motility. Previous studies have shown that activation of G proteins in bovine sperm transactivates the EGFR through protein kinase A (PKA) and Src (Etkovitz et al., 2009). Transactivation of EGFR through activation of stimulatory G protein βγ-subunits has been reported for another steroid membrane receptor, G protein-coupled estrogen receptor-1, (GPER-1) (Filardo et al., 2002). The βγ-subunits of the G proteins activate Src-related kinases, which in turn trigger matrix metalloproteinases to cleave pro-heparin-binding epidermal growth factor (pro-HB-EGF). The cleaved pro-HB-EGF then binds to EGFR, thus activating the receptor and increasing the activities of downstream MAPKs (Filardo and Thomas, 2005). EGFR has been shown to be involved in boar sperm motility (Oliva-Hernandez and Perez-Guiterrez, 2008) and EGF signaling has been demonstrated to be an important signaling pathway in high fertility bovine sperm in a comprehensive proteomic analysis (Peddinti et al., 2008). Peyton and Thomas (2011)

showed that inhibition of ErbB2, a member of the EGFR family, partially reduced the ability of zebrafish oocytes to respond to the endogenous MIS, 17α,20β-dihydroxy-4-pregnen-3-one (DHP). Previous work by Zhu et al., 2003 demonstrated that mPRα is the receptor that mediates this action of the MIS in zebrafish (Zhu et al., 2003). As mPRα also mediates the stimulatory effects of 20β-S on Atlantic croaker sperm motility, the possible role of ErbB2 and Erk1/2 pathways in progestin-stimulated sperm hypermotility in Atlantic croaker merits investigation.

Recent work by Tubbs et al (2011) showed that 20β-S is the major progestin produced by the southern flounder testes. Their findings also suggested that mPRα is the sperm receptor that mediates the effects of progestins on sperm hypermotility in this species as the abundance of mPRα on the plasma membrane fractions of flounder sperm was correlated to sperm motility (Tubbs et al., 2011). Moreover, the mechanism through which mPRα exerts progestin-stimulated sperm hypermotility appeared to be very similar to the mechanism previously described in Atlantic croaker (Tubbs and Thomas, 2009), as progestins activate G proteins and the inhibition of Acy also abolished progestin activated sperm hypermotility (Tubbs et al., 2011).

The southern flounder is a commercially valuable species with attractive aquaculture prospects (Smith and Denson, 2000). However, poor male reproductive performance, sperm quality and milt production are considered major causes of the relatively poor fertilization and reproductive success of captive spawns of southern flounder (Henderson-Arzapalo et al., 1988; Smith and Denson, 2000). Increased sperm motility resulting in increased fertilization success has previously been demonstrated in

Atlantic croaker (Tubbs and Thomas, 2009). While progestin stimulation of sperm hypermotility has clearly been shown (Tubbs et al., 2011), it has not been demonstrated that the increased flounder sperm swimming velocity influences male fertility. Similarly, there have been no reports on the effects of hormonal manipulation of male southern flounder on the quality of the milt produced and on fertilization success. Previous experiments by Tubbs and Thomas (2009) using Atlantic croaker showed that sperm swimming speeds were increased by an injection of a superactive gonadotropin-releasing hormone analog (LHRHa; des-Gly^{10},[d-Ala^{6}]LHRH (1–9) ethylamide) following an increase in mPRα protein expression on the sperm plasma membrane. Thus, due to the commercial value of southern flounder and its potential as an aquaculture species, hormonal manipulation of male flounder to increase fertilization success may be a practical method to improve the reproductive efficiency of captive broodstock.

It should be noted that direct evidence showing the involvement of mPRα mediation of sperm hypermotility in teleosts is lacking due to mature sperm being transcriptionally inactive. Thus, siRNA-induced loss-of-function experiments cannot be performed on sperm to further validate the role of mPRα in mediating progestin-stimulated sperm hypermotility. However, a recent study by Kelder et al (2010) identified two specific mPRα agonists that showed similar affinities as progesterone for recombinant human mPRα. These compounds displayed mPRα agonist activities in G-protein and MAPK activation assays and did not activate nuclear progestin receptors. As Org OD 02-0 is a specific agonist for mPRα, it is a powerful tool for validating that

progestin-activated sperm hypermotility is initiated by progestin binding to mPRα on the plasma membrane of teleost sperm.

Another largely unknown area of male gamete physiology is how environmental stressors (e.g. hypoxia and ocean acidification) affect it. Seasonal hypoxia has greatly increased in many estuaries and coastal regions such as the northern Gulf of Mexico over the past 30 years due to increased anthropogenic inputs of nutrients (Rabalais et al., 2007; Raymond et al., 2008). The long-term effects of recurring hypoxia exposure on marine ecosystems and valuable fisheries resources are unknown. Previous laboratory studies have shown that chronic exposure of male Atlantic croaker to hypoxia (dissolved $O_2 < 2$ mg/L) resulted in reduced gonad size, disruption of the endocrine system, including decreased circulating endogenous 20β-S levels, a decrease in the abundance of mPRα on sperm plasma membranes, and decreased sperm motility, an important determinant of sperm quality (Thomas and Rahman, 2009). These findings corroborate the findings of Thomas et al (2007) in a Florida estuary and more recently, from another study in the northern Gulf of Mexico (Thomas and Rahman, 2011). However, sperm motility analysis has never been performed in the field. Sperm motility analyses in the Gulf of Mexico would allow a direct correlation of the effects of seasonal hypoxia on an important functional aspect of male fertility as well as complement the previous studies cited.

Since the onset of the Industrial Revolution, the pH level of the world oceans has dropped by ~ 0.1 units, with some estimates suggesting that oceanic pH may continue to drop by ~ 0.4 units by the year 2100 (Caldeira and Wickett, 2003; Raven et al., 2005; Blackford and Gilbert, 2007). Studies indicate a decrease of ocean pH levels by 0.4 pH

units will have detrimental effects on physiological processes in a wide variety of species and ecosystems (Harley et al., 2006; Doney et al., 2009; Fabry et al., 2008). Recent interest has been turned to examining the effects of ocean acidification on the earliest life history stages of marine organisms – including external fertilization and larval development – that are highly susceptible to environmental changes. These stages are also key life stages for successful recruitment and ensuring continued survival of the species (Cowen et al., 2000; Raven et al., 2005). However, there is little or no data on how ocean acidification may affect the sperm physiology and reproductive success of vertebrates. Hypoxic dead zones further exacerbate ocean acidification as hypoxia is caused by the respiration of the sinking organic matter, which in turn also releases CO_2, thus also acidifying the subsurface water (Cai et al., 2011). The northern Gulf of Mexico (GOM) is an area that experiences an annual seasonal dead zone that is both hypoxic as well as highly acidic (Wang et al., 2013). Thus, a study on the effects of ocean acidification on the sperm physiology of the Atlantic croaker, a teleost species that is ubiquitous in the Gulf of Mexico, is particularly relevant.

Overall Goals

The overall goal of this research was to verify the involvement of mPRα in mediating progestin-stimulated sperm hypermotility in the Atlantic croaker and southern flounder. Novel intracellular signaling pathways involved in progestin-stimulated sperm hypermotility in Atlantic croaker sperm were also explored. Furthermore, the role of mPRα in southern flounder fertility was examined. Finally, due to growing concerns

about ocean hypoxia and acidification, male Atlantic croaker reproductive physiology was examined, with a particular interest in whether low oxygen and pH levels have a detrimental effect on reproductive parameters.

Specific hypotheses:

1. Progestin-induced sperm hypermotility is mediated by novel membrane steroid receptor, mPRα.
2. The βγ-subunits of the G_{olf} protein associated with mPRα are involved in progestin signaling when activated, initiating the Pi3k/Akt/Pde and ErbB2/Mapk pathways.
3. The mPRα/Acy/cAMP signaling pathway first described in Atlantic croaker sperm is conserved in advanced fishes, and therefore a similar mechanism is involved in progestin-induced sperm hypermotility in southern flounder.
4. Progestin-induced sperm hypermotility is inducible in high motility southern flounder sperm with high expression of mPRα, resulting in improved fertility.
5. Flounder sperm motility and fertility can be improved by *in vivo* administration of a superactive GnRH analog (LHRHa).
6. Atlantic croaker reproductive physiology is sensitive to environmental insults and its reproductive parameters are affected by exposure to either low oxygen or pH levels.

Chapter 2: Activation of the Pi3k/Akt pathway and modulation of phosphodiesterase activity via membrane progestin receptor-alpha (mPRalpha) regulate progestin-initiated sperm hypermotility in Atlantic croaker

Abstract

Rapid progestin stimulation of sperm motility is a widely observed phenomenon in vertebrates but the mechanisms governing these effects are still poorly understood, especially in teleosts, amphibians and birds, which do not express CatSper. Here we show that progestin-stimulated sperm hypermotility in a teleost, Atlantic croaker (*Micropogonias undulatus*), is initiated though membrane progestin receptor-alpha (mPRα, also known as Paqr7b), and involves activation of the Pi3k/Akt pathway and increased phosphodiesterase (Pde) activity. The specific mPRα agonist, 10-ethenyl-19-norprogesterone (Org OD 02-0) mimicked the stimulatory actions of the endogenous progestin in this species, 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S), on sperm motility. Inhibition of Pi3k (1 nM Wortmannin; 25 μM LY294002) and Akt (25 μM ML-9) effectively abolished progestin-initiated sperm hypermotility. Surprisingly, treatment with the PDE inhibitors Cilostamide (100 nM) and Rolipram (1 μM) also blocked progestin stimulation of sperm motility. Whereas treatment with 20β-S increased sperm Pde activity, pretreatment with Wortmannin eliminated this response to the progestin and also resulted in elevated cAMP levels, indicating that Pdes are at least partially under

Pi3k/Akt control. The results suggest that mPRα-mediated progestin stimulation of sperm motility in croaker through Pi3k/Akt is dependent upon maintenance of Pde activity and a reduction in internal cAMP concentrations. However, a previous study showed that progestin stimulation of sperm hypermotility in this species is also dependent upon membrane adenylyl cyclase (Acy) activation and increased intrasperm cAMP concentrations. Collectively, these findings indicate that progestin-mediated hypermotility through mPRα in teleost sperm involves modulation of intracellular cAMP concentrations through multiple signaling pathways.

Introduction

Rapid progestin actions at nanomolar concentrations on sperm physiology have been described in a variety of vertebrate species including the induction of hypermotility in teleost fish (Thomas, 2003; Tubbs and Thomas, 2009; Tubbs et al., 2011) and hyperactivation, chemotaxis towards the egg, and the acrosome reaction in mammalian sperm (Uhler et al., 1992; Park et al., 2011; Teves et al., 2006). The sperm of most teleost species with external fertilization are immotile in the seminal fluid and motility is triggered simultaneously in fish sperm by the marked change in external osmolality upon their release into the aquatic environment (Cosson et al., 2008). Pre-treatment of Atlantic croaker (*Micropogonias undulatus*) sperm with the teleost progestin hormone, 20β-S, causes concentration–dependent increases over the range of 5-200 nM in both sperm swimming speed (hypermotility) and in the percent hypermotile sperm upon activation

with a high osmolality activation buffer (Tubbs and Thomas, 2009; Thomas et al., 2004). Acquisition of the ability of Atlantic croaker sperm to become hypermotile likely occurs *in vivo* prior to sperm release into the seawater since the seminal fluid contains nanomolar concentrations of progestins (Tubbs and Thomas, 2009). However, progestin hormones produced by females and released in the ovarian fluid at spawning could also potentially participate in induction of hypermotility because the sperm are released in very close proximity to the eggs. The common features of progestin–induced fish sperm hypermotility have not been well delineated because it has only been reported in a few teleost species. Hypermotility is characterized by an increase in sperm swimming speed which in some teleost species such as Atlantic croaker is accompanied by an increase in the rate of turning (Tubbs and Thomas, 2009). Thus, the characteristics of sperm hypermotility in teleosts differ from those of hyperactivation in mammalian sperm (Ho and Suarez, 2001). However, details of the mechanisms through which progestins mediate hypermotility in fish sperm remain largely elusive (Thomas, 2003; Tubbs and Thomas, 2009; Tubbs et al., 2011).

Membrane progestin receptors have been biochemically characterized on spotted seatrout (Thomas et al., 1997), Atlantic croaker (Thomas et al., 2005) and southern flounder (Tubbs et al., 2011) sperm plasma membranes and are hypothesized to be the progestin-binding moieties that mediate sperm hypermotility in these species. Recent evidence suggests that a novel membrane receptor originally identified on spotted seatrout ovaries (Zhu et al., 2003), which belongs to the progestin and adipoQ receptor (PAQR) family (Thomas et al., 2007), named membrane progestin receptor alpha (mPRα,

also known as Paqr7b), is a potential candidate for the receptor mediating progestin sperm hypermotility. The mPRα protein is expressed on the plasma membrane and localized to the sperm midpiece and flagella in teleost fish (Tubbs and Thomas, 2009; Tubbs et al., 2011; Zhu et al., 2003) and humans (Thomas et al., 2009). Moreover, there is a positive correlation between sperm swimming speed and the abundance of mPRα on the plasma membranes of fish sperm (Tubbs and Thomas, 2009; Tubbs et al., 2011). However, there has been considerable debate over the exact role of mPRα in progestin signaling in vertebrate cells (Thomas, 2012). Although it is now widely accepted that mPRs are high affinity, specific progesterone receptors (Thomas, 2012; Smith et al., 2008; Gellerson et al., 2009), the importance of G proteins as intermediaries in progesterone activation of signaling pathways through these receptors remains controversial and incompletely resolved. While G protein activation has been demonstrated through all five mPR subtypes in a broad variety of vertebrate cells (Thomas et al., 2007; Pang and Thomas, 2011; Pang et al., 2013), progestin signaling through recombinant mPRs has also been observed in a heterologous yeast expression system which do not express G proteins (Smith et al., 2008). Moreover, a physiological role of mPRs in mediating the many functions of progestins in reproductive and nonreproductive tissues has not been clearly demonstrated. For example, definitive evidence that progestin induction of sperm hypermotility in teleosts is mediated through mPRα is currently lacking.

The finding that mPRα is coupled to a stimulatory olfactory G protein (G_{olf}) in Atlantic croaker sperm is consistent with the results of studies showing that progestin

treatment causes a rapid increase in cAMP production by sperm membranes (Tubbs and Thomas, 2009). A potential mechanism by which progestins could induce hypermotility in croaker sperm is through activation of the G_{olf} α-subunit resulting in increases in the activity of membrane-bound adenylyl cyclase (Acy) and elevated cAMP levels which in turn may cause mononucleotide-gated Ca^{2+} channels to open, leading to an increase in intrasperm free Ca^{2+} concentrations (Tubbs and Thomas, 2009). In support of this suggestion, treatment with an Acy inhibitor was shown to block progestin-induced hypermotility and the increase in cAMP levels (Tubbs and Thomas, 2009).

In marked contrast to the clear evidence for an involvement of the stimulatory G_{olf} α-subunit in progestin induction of sperm hypermotility in teleosts, comparable information on the possible role of the G protein βγ-subunit in mediating this sperm function is lacking. The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is one of the signaling cascades commonly associated with βγ-subunit signaling (Stephens et al., 1997; Djordjevic and Driscoll, 2002). The PI3K/AKT pathway has been implicated in mediating male fertility in various tetrapods; the PI3K/AKT pathway has been reported to regulate sperm viability in boar sperm (Aparicio et al., 2007) and the pathway is suggested to regulate sperm motility in hamsters (NagDas et al., 2002) and chickens (Ashizawa et al., 2008). On the basis of the results of a previous study showing that induction of meiotic maturation in Atlantic croaker oocytes by the endogenous progestin, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), is blocked by pretreatment with PI3K and PDE inhibitors, it was suggested that Pi3k activates Pdes in croaker oocytes that in turn promote meiotic maturation by decreasing cAMP levels (Pace and Thomas, 2005).

These findings raise the possibility that progestin stimulation of gamete maturation through mPRα in male Atlantic croaker also involves activation of Pi3k/Akt signaling. In support of this, preliminary evidence was obtained in croaker sperm (Tan and Thomas, 2010) and more recently in human sperm (Sagare-Patil et al., 2012) for activation of Akt by progestins at low (100 nM) and high (5-10 μM) concentrations, respectively. However, the precise role of the βγ-subunits of the G protein associated with mPRα in progestin regulation of hypermotility in croaker sperm has not been examined. For example, it is not known whether the effects of Pi3k/Akt signaling are mediated through modulation of Pdes. Moreover it is not known whether there are any interactions between the signaling cascades initiated by these G protein α- and βγ-subunits through mPRα in croaker sperm.

Vertebrate sperm are good models for investigating nongenomic steroid actions in isolation because sperm are generally considered to be transcriptionally inactive. However, this characteristic of sperm precludes the use of gene silencing procedures to knockdown expression of mPRα and other receptors for loss-of-function experiments in order to confirm their physiological roles. We recently identified two synthetic progestins that displayed potent mPRα agonist activities in G protein and mitogen-activated protein kinase (MAPK) activation assays in mPRα-transfected cells, but were inactive in a nuclear progesterone receptor transactivation assay (Kelder et al., 2010). Thus, these compounds are powerful tools for identifying progestin functions mediated by mPRα. Therefore, one of these compounds, 10-ethenyl-19-norprogesterone (Org OD 02-0), was

used in the present study to investigate whether progestin activation of croaker sperm hypermotility is mediated through mPRα.

In the present study, we tested the hypothesis that progestin-mediated sperm hypermotility in Atlantic croaker is partially mediated through the Pi3k/Akt pathway and its downstream effectors via mPRα. In addition, the hypothesis that the βγ-subunits of the G protein coupled to mPRα mediate opposite progestin actions on cAMP levels to stimulate sperm motility than those induced through the G protein α-subunit was also tested. Signaling pathways mediating progestin stimulation of sperm hypermotility in croaker can be investigated in the absence of progestin signaling associated with the acrosome reaction because teleost sperm do not undergo this process. CatSper, the calcium channel mediating progesterone induction of sperm motility in mammals (Lishko et al., 2011; Strunker et al., 2011), is not present on the sperm of teleosts, birds and amphibians (Cai and Clapham, 2008). Therefore, the CatSper-independent Atlantic croaker sperm model represents an alternative mechanism of progestin induction of sperm motility that may be applicable to a wide variety of nonmammalian vertebrates.

Materials and Methods

Chemicals

The Atlantic croaker progestin hormone, 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) was purchased from Steraloids (Newport, RI). The synthetic progestin, 10-ethenyl-19-norprogesterone (Org OD 02-0) was a gift from Organon (Oss, the

Netherlands). The Akt and P-Akt antibodies were purchased from Cell Signaling Technology (Danvers, MA). All inhibitors were purchased from Enzo Life Sciences (Farmington, NY). The cAMP measurement kit was purchased from Cayman Chemical (Ann Arbor, MI). The Pde activity assay kit was purchased from Mediomics (St. Louis, MO). All other chemicals and reagents were obtained from Sigma unless otherwise noted.

Animals

Adult Atlantic croaker were purchased in the fall from local bait shops. Fish were acclimated to the laboratory for two months before use in 12000 L recirculating tanks at 22 – 24 °C and a photoperiod of 11 hr light, 13 hr dark to promote and maintain gonadal development. All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Milt collection

Milt was collected from fully mature male Atlantic croaker as described previously (Detweiler and Thomas, 1998). Briefly, the abdomen of the fish was wiped dry to prevent exposure to seawater that causes premature sperm activation. Gentle pressure was applied to the abdomen and the expressed milt was collected from the cloaca with a clean syringe and transferred to tubes that were kept on ice. Care was taken to prevent contamination of milt samples with urine, which would cause premature activation of sperm.

Preparation of sperm plasma membranes

Sperm membranes were isolated as previously described (Thomas at al., 1997) with minor modifications. Briefly, 3 – 5 mL of milt were diluted in 10 mL of ice-cold homogenization buffer [HAED; 25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA (pH 7.6)] and centrifuged at 1000 × *g* to isolate sperm from seminal fluid. Sperm were resuspended in 10 mL HAED with protease inhibitors (Merck, Darmstadt, Germany). Sperm suspensions were forced through a 23.5 gauge needle twice and sonicated at medium power for 6 s on ice. Samples were then centrifuged at 500 × *g* for 10 min at 4 °C to remove the nuclear fraction. The supernatant was transferred to a clean tube and centrifuged at 17000 × *g* for 20 min at 4 °C to pellet the cell membrane fraction. Isolated sperm membranes were used immediately or stored at -80 °C. Protein concentrations of membrane preparations were determined using a Bradford protein assay (Bio-Rad, Hercules, CA).

Sperm motility analyses

Sperm motility experiments were performed as described previously (Detweiler and Thomas, 1998) with minor modifications. Briefly, croaker milt was diluted 200-fold and preincubated in a 340 mOsm/kg physiological saline (predilutant: 160 mM NaCl, 8.6 mM KCl, 3 mM $CaCl_2$·2H2O, 10 mM $NaHCO_3$, 1.3 mM Na_2HPO_4, 2.5 mM $MgCl_2 \cdot 6H_2O$, 5 mM D-glucose, pH 7.8) with steroid (20β-S or Org OD 02-0) or vehicle (EtOH, 1%) for 1 min at room temperature. A 2.5 µL aliquot of each sperm suspension was added to 25 µL hyperosmotic (640 mOsm/kg) activating solution (320 mM NaCl, 17.2 mM KCl, 20 mM $NaHCO_3$, 2.6 mM Na_2HPO_4, 5 mM $MgCl_2 \cdot 6H_2O$, 10 mM D-glucose, pH 7.8) on a microscope slide. It should be noted that the increase in external

osmolality is the sole stimulus triggering motility of marine fish sperm, because replacement of the ions with a hypertonic solution of glucose or other non-ionic osmolytes has been shown to cause the same activation of sperm motility in numerous marine species (Cosson et al., 2008). A coverslip was placed on the slide and sperm motility was recorded for 1 min using a dark field microscope connected to a computer capable of recording high quality videos. Each experiment was recorded using a charge-coupled device camera (Cohu Electronics, San Diego, CA) and digital recording software (Pinnacle, Mountain View, CA). Sperm swimming speed (average path velocity) was determined using CellTrak motion analysis software (Motion Analysis Corp., Santa Rosa, CA) for typically 15-25 sperm for each treatment in a single trial and the average velocity calculated. Each treatment trial was repeated three times in a single experiment and each study consisted of at least three experiments with sperm from different donors (see figure legends for more details). The results shown represent the composite of means of the average swimming speeds ± SEM. For experiments with inhibitors, milt was diluted in physiological saline as described above and preincubated with the inhibitor or vehicle (dimethylsulfoxide, DMSO; 0.1%) for 30 min before treatment with steroid or vehicle for 1 min and activation with artificial seawater. Concentrations of inhibitors were used that did not compromise basal sperm motility. Sperm motility was recorded as described above. Percent hypermotile sperm was determined from the percent of motile sperm that were hypermotile (i.e. increased swimming speeds and rates of turning). For experiments employing inhibitors of the PI3K/AKT/PDE pathway, the average number of motile

sperm in the field of view was also calculated to determine whether the inhibitor treatments were cytotoxic.

Western blot analyses

Approximately 10 μg of membrane protein was added to loading buffer [0.5 M Tris-HCl, 10% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue, 10% glycerol] and resolved on 10% SDS-PAGE gels. After transfer to nitrocellulose membranes, membranes were blocked in a solution containing 5% nonfat milk, 0.1% Tween 20 in PBS [136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4 (pH 7.4)]. Membranes were rinsed with PBS and incubated overnight at 4 °C with primary antibodies directed towards P-Akt or total Akt, (Cell Signaling, Boston, MA; 1:1000) in a blocking solution containing 5% BSA and 0.1% Tween 20 in PBS. Membranes were rinsed with PBS and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam, Cambridge, UK) in blocking solution containing 5% nonfat milk and 0.1% Tween 20 in PBS for 1 hr. Proteins were then visualized using SuperSignal West-Pico chemiluminescent substrate (Thermo Fisher Scientific Inc. Rockford, IL) and exposed to X-ray film (GE Healthcare, Buckinghamshire, UK). P-Akt expression was normalized to total Akt using ImageJ software (NIH) to obtain relative densitometries.

Measurement of cAMP production by croaker sperm membranes

Sperm cell membranes were resuspended in buffer [75 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EDTA (pH 7.6)] to a total protein concentration of 1 mg/mL. To investigate the effects of phosphodiesterase inhibitors on the cAMP levels, sperm cell

membrane preparations were preincubated with a combination of 100 nM Cilostamide and 1 μM Rolipram for 30 min. Membrane preparations (100 μL) were added to assay buffer [0.2 mM ATP, 10 nM GTP, 0.5 mM phosphoenolpyruvate, 20 μg pyruvate kinase] containing either 20 nM 20β-S, 20 nM Org OD 02-0, 10 μM forskolin (Acy activator) or vehicle. Reactions were allowed to proceed for 1 min at room temperature and then samples were boiled for 5 min and centrifuged at 14,000 × *g* for 10 min. Supernatants were obtained, diluted 10- to 20-fold, and cAMP concentrations were determined using a commercial cAMP enzyme immunoassay kit following manufacturer's instructions (Cayman Chemincal, Ann Arbor, MI). Data were normalized to mean control values to account for donor variability.

Measurement of phosphodiesterase activity in croaker sperm

About 1 mL of milt was collected from croaker and placed immediately on ice. Milt was suspended in 5 mL of ice-cold homogenization buffer [HAED; 25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA (pH 7.6)] supplemented with protease inhibitors (Merck, Darmstadt, Germany). Sperm suspensions were sonicated at medium power for 6 s on ice. Samples were then centrifuged at 500 × *g* for 10 min at 4 °C to remove the nuclear fraction. The supernatant was then incubated with either 10 nM Wortmannin or vehicle for 30 min at room temperature. Phosphodiesterase activity was measured using the Bridge-It cAMP Pde Assay kit (Mediomics, St. Louis, MO). Sperm suspensions were mixed with the reaction mixture according to the manufacturer's instructions containing either 20 nM of 20β-S or vehicle and allowed to proceed for 30 min at room temperature. A stop solution was added and

samples were mixed well and left at room temperature for 5 min. Assay solution was added and the mixture was left at room temperature for 30–60 min before measuring fluorescence with a fluorescence plate reader. Data were normalized to mean control values to account for donor variability.

Statistical analyses

For all experiments, data are presented as means ± SEM. Statistical significance was determined using one-way ANOVA and Dunnett's or Tukey's multiple comparison post-tests using GraphPad Prism 5 Software (San Diego, CA).

Results

Effects of the selective mPRα agonist Org OD 02-0 on sperm hypermotility

The role of mPRα (Paqr7b) in mediating progestin induction of Atlantic croaker sperm hypermotility was examined by testing whether incubation with 20 nM of the selective mPRα agonist Org OD 02-0 for 1 min mimicked the stimulatory actions of 20β-S. Treatment with 20 nM of the endogenous progestin hormone, 20β-S, or Org OD 02-0 significantly increased the average sperm swimming speed (105.1 ± 2.1 μm/s, $P<0.05$ and 103.8 ± 5.8 μm/s, $P<0.05$ respectively), compared to vehicle-treated controls (86.4 ± 3.1 μm/s; Fig. 2.1).

Effects of specific inhibitors of the PI3K/AKT pathway on progestin-induced sperm hypermotility

The effect of the specific PI3K inhibitor, Wortmannin, on Atlantic croaker sperm motility was tested by preincubating sperm with the compound for 30 min prior to treatment with progestins or vehicle. Treatment with 20β-S increased sperm swimming speed (117.6 ± 3.6 μm/s, $P<0.05$; Fig. 2.2A) and the percent of hypermotile sperm (84.2 ± 2.1 %, $P<0.001$; Fig. 2.2B), compared to their vehicle-treated controls (103.6 ± 3.0 μm/s and 56.9 ± 2.3 %, respectively). Preincubation of the same pool of sperm with 1 nM Wortmannin for 30 min resulted in abrogation of the 20β-S-induced hypermotility (Fig. 2.2A, B). Treatment of croaker sperm with Org OD 02-0 also resulted in an increase in sperm swimming speed (129.5 ± 3.9 μm/s, $P<0.05$; Fig. 2.2C) compared to controls (110.4 ± 4.5 μm/s). Preincubation of the same pool of sperm with 1 nM Wortmannin for 30 min resulted in almost complete attenuation of the Org OD 02-0-induced sperm hypermotility (Fig. 2.2C). None of the treatments significantly decreased the number of motile sperm detected in a single field of view compared to controls in the 20β-S stimulation of hypermotility experiment (20β-S: 15.4 ± 3.4, Wortmannin: 17.8 ± 2.4, 20β-S + Wortmannin: 19.1 ± 4.5, controls: 22.7 ± 2.0, NS), and also in the Org OD 02-0 stimulation of hypermotility experiment (Org OD 02-0: 27.1 ± 2.6, Wortmanin: 19.8 ± 3.0, Org OD 02-0 + Wortmanin: 29.0 ± 2.9, controls: 27.4 ± 4.5, NS).

These results were confirmed by preincubation of croaker sperm with another specific PI3K inhibitor, LY294002. As observed before, 20β-S increased sperm swimming speed (118.6 ± 4.9 μm/s, $P<0.05$; Fig. 2.3A) and the percent of hypermotile sperm (75.6 ± 3.4 %, $P<0.001$; Fig. 2.3B), compared to their vehicle-treated controls (98.3 ± 4.4 μm/s and 47.8 ± 5.1 %, respectively). Preincubation of sperm with 25 μM

LY294002 for 30 min abolished the 20β-S-induced hypermotility (Fig. 2.3A, B). Treatment of croaker sperm with Org OD 02-0 also increased the average sperm swimming speed (122.7 ± 3.4 μm/s, $P<0.05$; Fig. 2.3C) compared to controls (108.3 ± 3.9 μm/s). Pretreatment of the same pool of sperm with 25 μM LY294002 for 30 min eliminated the stimulatory effect of Org OD 02-0 on sperm motility (Fig. 2.3C). None of the treatments significantly decreased the number of motile sperm detected in a single field of view compared to controls in the 20β-S stimulation of hypermotility experiment (20β-S: 21.7 ± 3.4, LY294002: 21.7 ± 3.2, 20β-S + LY294002: 19.7 ± 4.5, controls: 21.8 ± 3.5, NS), and also in the Org OD 02-0 stimulation of hypermotility experiment (Org OD 02-0: 25.8 ± 3.0, LY294002: 23.9 ± 4.1, Org OD 02-0 + LY294002: 21.1 ± 4.9, controls: 25.7± 4.7, NS).

The effect of a specific AKT inhibitor, ML-9, was also tested on Atlantic croaker sperm motility. Treatment of croaker sperm with 20β-S increased sperm swimming speed (117.8 ± 2.7 μm/s, $P<0.001$; Fig. 2.4A) and the percent of hypermotile sperm (80.4 ± 1.7 %, $P<0.001$; Fig. 2.4B) compared to their vehicle-treated controls (99.0 ± 3.1 μm/s and 49.0 ± 1.8 %, respectively). Preincubation of sperm with 25 μM ML-9 for 30 min eliminated the 20β-S-induced hypermotility (Fig. 2.4A, B). Treatment of croaker sperm with Org OD 02-0 also increased sperm swimming speeds (124.0 ± 3.0 μm/s, $P<0.05$; Fig. 2.4C) compared to controls (106.8 ± 3.2 μm/s). Pretreatment of sperm with 25 μM ML-9 for 30 min also abrograted the Org OD 02-0 stimulatory effect on sperm motility (Fig. 2.4C). None of the treatments significantly decreased the number of motile sperm detected in a single field of view compared to controls in the 20β-S stimulation of

hypermotility experiment (20β-S: 15.4 ± 2.5, ML-9: 12.0 ± 2.2, 20β-S + ML-9: 14.8 ± 2.3, controls: 13.3 ± 1.2, NS), and also in the Org OD 02-0 stimulation of hypermotility experiment (Org OD 02-0: 19.0 ± 2.4, ML-9: 22.6 ± 4.4, Org OD 02-0 + ML-9: 26.1 ± 5.6, controls: 21.9 ± 3.7, NS).

Identification of Akt in croaker sperm and effects of PI3K inhibitor treatment on progestin activation of Akt .

Immunoreactive bands of the correct sizes for Akt and phosphorylated Akt (P-Akt) were detected in croaker sperm membrane samples by Western blot analysis using antibodies generated against Akt and Akt phosphorylated at Ser473, respectively. Treatment with 20β-S increased the amount P-Akt in the sperm membrane preparations (Fig. 2.5). The effect of Wortmannin on Atlantic croaker sperm Akt phosphorylation was assessed by preincubating a pool of sperm with either vehicle or 10 nM Wortmannin and detecting the amount P-Akt by Western blot analysis. The amount of P-Akt was normalized to total Akt for comparison between sperm treated with either vehicle or 20β-S. Akt phosphorylation was increased by 20β-S (Fig. 2.5) by 45.4 ± 10.0 % compared to control. Preincubation of sperm with 10 nM Wortmannin for 30 min resulted in abrogation of the progestin-induced Akt phosphorylation (Fig. 2.5).

Effects of specific inhibitors of PDEs on progestin-induced sperm hypermotility

The effect of inhibitors of PDE3 (Cilostamide) and PDE4 (Rolipram) on sperm motility was investigated by preincubating sperm with either compound for 30 min prior to exposure to progestins or vehicle. Both 20β-S (Fig. 2.6A) and Org OD 02-0 (Fig. 2.6B) increased sperm swimming speed 125.8 ± 4.7 μm/s and 130.8 ± 5.5 μm/s,

respectively, compared to their respective vehicle-treated controls (101.0 ± 6.6 μm/s and 110.0 ± 2.9 μm/s, $P<0.05$; Fig. 2.6A, B). Preincubation of sperm with 100 nM Cilostamide for 30 min resulted in abrogation of the 20β-S-induced hypermotility (Fig. 2.6A, B). None of the treatments significantly decreased the number of motile sperm detected in a single field of view compared to controls in the 20β-S stimulation of hypermotility experiment (20β-S: 7.5 ± 2.2, Cilostamide: 5.4 ± 1.5, 20β-S + Cilostamide: 5.6 ± 1.8, controls: 4.8 ± 0.8, NS), and also in the Org OD 02-0 stimulation of hypermotility experiment (Org OD 02-0: 22.1 ± 2.8, Cilostamide: 15.9 ± 3.4, Org OD 02-0 + Cilostamide: 20.4 ± 4, controls:15.2 ± 2.4, NS).

Similar results were observed in sperm pretreated with Rolipram. Both 20β-S (Fig. 2.6C) and Org OD 02-0 (Fig. 2.6D) increased sperm swimming speed 126.9 ± 3.5 μm/s ($P<0.05$) and 115.8 ± 6.0 μm/s ($P<0.01$), respectively, compared to their respective vehicle-treated controls (102.10 ± 6.148 μm/s and 96.8 ± 2.7 μm/s). Preincubation of sperm with 1 μM Rolipram for 30 min abolished the Org OD 02-0-induced hypermotility (Fig. 2.6C, D). None of the treatments significantly decreased the number of motile sperm detected in a single field of view compared to controls in the 20β-S stimulation of hypermotility experiment (20β-S: 17.2 ± 3.2, Rolipram: 15.1 ± 2.8, 20β-S + Rolipram: 13.0 ± 3.6, controls: 14.6 ± 3.3, NS), and also in the Org OD 02-0 stimulation of hypermotility experiment (Org OD 02-0: 18.0 ± 2.5, Rolipram: 13.6 ± 2.0, Org OD 02-0 + Rolipram: 13.9 ± 1.8, controls: 15.7 ± 2.4, NS).

Effects of co-treatment with PI3K or PDE inhibitors with progestins on production of cAMP by croaker sperm membranes

The effect of Pde inhibition on cAMP production by Atlantic croaker sperm membranes was examined by preincubating a pool of sperm membranes with either vehicle, or 100 nM Cilostamide and 1μM Rolipram for 30 min before assaying for cAMP with a commercially available kit. Forskolin was used as a positive control as it has previously been shown to act as a Acy activator (Tubbs and Thomas, 2009). The study was conducted without the use of 3-isobutyl-1-methylxanthine (IBMX), a nonselective PDE inhibitor.

Membrane samples pretreated with a combination of the two PDE inhibitors, 100 nM Cilostamide and 1μM Rolipram, produced significantly higher amounts of cAMP in vehicle-treated (232.8 ± 42.3 %, $P<0.05$), 20 nM 20β-S (255.4 ± 48.5 %, $P<0.01$) or 20 nM Org OD 02-0 (239.2 ± 13.1 %, $P<0.05$) treatment groups compared to the vehicle pretreated alone control (normalized to 100%) (Fig. 2.7A). In membrane samples pretreated with vehicle alone, both progestin treatments did not cause significant increases in cAMP production when compared to vehicle-treated control (Fig. 2.7A).

The effect of Pi3k inhibition on cAMP production by Atlantic croaker sperm membranes was examined by preincubating a pool of sperm membranes with either vehicle, or 10 nM Wortmannin for 30 min before assaying for cAMP. In membrane samples pretreated with Wortmannin, treatment with either 20 nM 20β-S or 20 nM Org OD 02-0 caused a significant increase in cAMP production (220.2 ± 4.8 % and 219.7 ± 19.8 %, respectively) compared to the vehicle treated control ($P<0.001$; Fig. 2.7B). However, in membrane samples pretreated with vehicle, both 20 nM 20β-S and 20 nM

Org OD 02-0 did not significantly increase cAMP production by the croaker sperm plasma membrane fractions (Fig. 2.7B).

Effects of PI3K inhibitor treatment on progestin-induced increase in Pde activity in croaker sperm

The effect of Pi3k inhibition on Pde activity was tested by preincubating sperm homogenates with either 10 nM Wortmannin or vehicle for 30 min before assaying Pde activity with a commercially available kit. Samples pretreated with vehicle and then treated with 20 nM 20β-S showed a significant increase in Pde activity (114.4 ± 3.2%) compared to control (normalized to 100%) (Fig. 2.8). Preincubation of sperm homogenates with 10 nM Wortmannin abolished the progestin-induced increase in Pde activity (Fig. 2.8).

Discussion

In this study, we demonstrate that progestin-induced sperm hypermotility in Atlantic croaker is mediated through mPRα and the Pi3k/Akt pathway. These results provide the first evidence for a role for the Pi3k/Akt pathway in the regulation of sperm motility in teleost fish and also suggest a plausible mechanism through which the βγ-subunits of the G protein coupled to mPRα (G_{olf}) may influence sperm function. Although the results of our previous studies had suggested that mPRα mediates progestin induction of sperm hypermotility, direct evidence for this was lacking because knock-down of mPRα expression for loss-of-function experiments cannot be conducted in sperm

as they are transcriptionally inactive. The discovery of selective mPRα agonists provides a valuable alternative approach for investigating the functions of this receptor in vertebrate sperm. In the current study, we show that the selective mPRα agonist, Org OD 02-0 mimics the stimulatory effects of the endogenous Atlantic croaker progestin, 20β-S, on sperm motility, indicating that progestin-mediated sperm hypermotility in this species is mediated by mPRα. This is consistent with previous results that suggest this progestin action is not mediated through the nuclear progestin receptor (nPR) because the nPR agonist, R5020, did not stimulate sperm motility in two teleost species, the Atlantic croaker (Tubbs and Thomas, 2009) and southern flounder (Tubbs et al., 2011). The observation that there is a positive correlation between sperm motility and the abundance of the mPRα protein on croaker (Tubbs and Thomas, 2009) and southern flounder sperm Tubbs et al., 2011) further supports this role for mPRα. Collectively, these results provide strong evidence that progestin stimulation of sperm hypermotility is primarily induced through mPRα in Atlantic croaker.

PI3K and AKT have previously been detected in the sperm of several mammalian species and also in chickens (NagDas et al., 2002; Ashizawa et al., 2008; Aparicio et al., 2005; Luconi et al., 2001). The identification of Akt in the present study in the sperm of teleost fish, which diverged from the tetrapod lineage over 200 million years ago, suggests that PI3K/AKT signaling is a fundamental, conserved characteristic of vertebrate sperm. However, its precise role in regulating sperm functions in tetrapods remains elusive. Treatment with the PI3K inhibitor LY294002 increased motility of human (Luconi et al., 2001) and boar (Aparicio et al., 2005) sperm, whereas treatment

with another inhibitor, Wortmannin, inhibited capacitation and motility of hamster sperm (NagDas et al., 2002). The functions of the PI3K/AKT pathway are also uncertain in avian sperm because both inhibitory effects and no effects of LY294002 treatments on the motility of chicken sperm have been reported (Ashizawa et al., 2008; Lemoine et al., 2009). Some of these differing results have been attributed to the PI3K inhibitor used, because the stimulatory effects of LY294002 on human sperm capacitation and motility were not observed with Wortmannin (du Pleiss et al., 2004, Nauc et al., 2004), and these two unrelated inhibitors have different effects on intracellular calcium concentrations (Nauc et al., 2004). Therefore, both PI3K inhibitors and an AKT inhibitor, ML-9 were used in the present study to clarify the role of the Pi3k/Akt pathway in the regulation of sperm motility in Atlantic croaker. The same effects on sperm motility were observed after pretreatment with all three inhibitors. None of the treatments significantly altered basal (unstimulated) sperm motility, whereas all three inhibitors blocked progestin-induced hypermotility. Thus the results clearly support our hypothesis that progestin induction of hypermotility in Atlantic croaker involves activation of the Pi3k/Akt pathway. This is further supported by finding that progestin treatment of croaker sperm increases phosphorylation of Akt, which is blocked by pretreatment with Wortmannin. Interestingly, progesterone has recently been reported to have similar actions in human sperm (Sagare-Patil et al., 2013), which suggests the involvement of PI3K/AKT signaling in progestin stimulation of motility may be widespread in vertebrate sperm.

Arguably the most interesting discovery of the present study is that PDE3 and PDE4 inhibitors block croaker sperm hypermotility rather than activating it as typically

observed with the motility of mammalian sperm (Fisch et al., 1998; Dimitriadis et al., 2008). Although the beneficial effects of many PDE inhibitors on sperm motility and fertility in humans and other mammalian species is widely recognized (Dimitriadis et al., 2008), detailed knowledge of the characteristics of sperm PDEs and their functions in mature sperm is lacking (Dimitriadis et al., 2008; Vasta et al., 2005). The localization of PDE4 primarily on the midpiece of human sperm (Fisch et al., 1998) and PDE3A on the postacrosomal region of the sperm head (Lefievre et al., 2002)] suggest they may have roles in sperm motility and capacitation, respectively. Although the Pdes in fish sperm have not been identified, the present results with Cilostamide and Rolipram suggest the presence of Pde3 and Pde4 in croaker sperm and their involvement in progestin-induced sperm hypermotility. This role of Pdes is further supported by the observation that progestin treatment increases Pde activity in croaker sperm. The finding that Pde activity also rapidly increases in sperm after its activation in another teleost species, rainbow trout (Morisawa and Ishida, 1987), suggests that an increase in Pde activity is commonly associated with sperm activation in this vertebrate group.

The role of PI3K/AKT signaling in regulating sperm motility remains controversial in part because information is currently lacking on the downstream targets of PI3K/AKT activation. Moreover, the interpretation of PI3K/AKT signaling results is complicated by its proposed involvement in the regulation of a wide variety of other sperm functions in tetrapods including viability (Aparicio et al., 2007), protection against oxidative stress and apoptosis (Koppers et al., 2011), and the acrosome reaction (Lemoine et al., 2009). The present study indicates a plausible mechanism by which

Pi3k/Akt signaling can influence teleost sperm hypermotility by altering Pde actvity and cAMP levels. The data demonstrate that the PI3K inhibitor, Wortmannin, prevents hypermotility of croaker sperm by attenuating the progestin-induced increase in Pde activity and by causing an increase in cAMP production in the presence of progestins. Taken together our results show that progestin activation of the Pi3k/Akt pathway through mPRα causes upregulation of Pde activity resulting in a rapid modulation of cAMP levels and croaker sperm hypermotility. Pde4-dependent sperm motility, though, is likely to be mediated through a different signaling pathway, since this PDE is regulated by cAMP in mammals (Conti et al., 2003).

The finding that progestins increase cAMP production when Pde activity is inhibited is consistent with previous results showing that 20β-S increases cAMP production by croaker sperm membranes that have been pretreated with a nonselective PDE inhibitor, IBMX (Tubbs and Thomas, 2009). The demonstration in that study that the 20β-S-induced increase in cAMP production and sperm hypermotilty was mimicked by treatment with an activator of membrane adenylyl cyclase (Acy), forskolin, and blocked by pretreatment with the Acy inhibitors 2,5-dideoxyadenosine (dd-Ado) and SQ22536 clearly implicates activation of Acy in this progestin action. The discovery that 20β-S activates a stimulatory G protein, G_{olf}, in croaker sperm and that mPRα is coupled to it provides a likely mechanism through which the progestin activates Acy (Tubbs and Thomas, 2009). A correlation between croaker sperm hypermotility and increased cAMP production was anticipated because 20β-S had previously been shown to increase sperm cAMP levels in croaker (Thomas, 2003) and there is extensive evidence that an increase

in cAMP is required for motility of vertebrate sperm (Morisawa and Morisawa, 1990; Tash and Means, 1983; Fraser et al., 2005; Suarez and Ho, 2003; Wade et al., 2003; Harrison, 2003). Although these earlier results with croaker sperm appear to be inconsistent with the present findings, activation of both Acy and Pde has been observed during the initiation of sperm motility in another teleost species (Morisawa and Ishida, 1987; Morisawa and Morisawa, 1990). Collectively, our results suggest that both progestin-induced increases in sperm Acy and Pde activities are required for the initiation and maintenance of croaker sperm hypermotility.

PDEs such as PDE4 have critical roles in regulating cAMP signaling and desensitization as well as cAMP concentrations in discrete cellular compartments such as those close to the plasma membrane (Conti et al, 2003). Increased PDE activity may be responsible for the short duration (1 min) of elevated cAMP concentrations observed upon activation of rat sperm (Wade et al., 2003). The activity of a PDE highly expressed on mammalian sperm flagella, PDE1 (Vasta et al., 2005; Yan et al., 2001), is calmodulin-dependent (Lefievre et al., 2002). Calcium plays has a fundamental regulatory role in the activation of sperm motility in vertebrates (Suarez, 2008; Lesich et al, 2012; Darszon et al., 2011). Rapid oscillations of Ca^{2+} concentrations have been reported in hyperactivated mammalian sperm that are correlated with tail beat frequency (Suarez et al., 1993; Harper et al., 2004). Calcium oscillations in embryonic spinal neurons are accompanied by transient changes in cAMP concentrations (Gorbunova and Spitzer, 2002), suggesting an interdependency between oscillation patterns of Ca^{2+} and cAMP that could potentially mediate a wide diversity of specific responses (Zaccolo and Pozzan, 2003). Currently,

detailed information is lacking on the pattern of the cAMP response in mammalian sperm during hyperactivation and it is not known whether the Ca^{2+} oscillations in sperm are accompanied by oscillations in cAMP (Vasta et al., 2005). The finding that activation of both Acy and Pde are required for induction of croaker sperm hypermotility does suggest though that a specific pattern of cAMP changes is needed to initiate this response.

In conclusion, the results of the present study show that progestin-induced sperm hypermotility in Atlantic croaker is mediated through mPRα and the Pi3k/Akt pathway and involves activation of Pdes. Evidence is presented for the existence of multiple signaling pathways regulating progestin-induced teleost sperm motility, in which the βγ-subunits of the G protein coupled to mPRα (G_{olf}) work in tandem with a previously reported α-subunit function (Tubbs and Thomas, 2009; Tubbs et al., 2011) to regulate cAMP levels. The finding that both Acy and Pde are activated in croaker sperm suggests that progestin-induced hypermotility may be dependent on rapid cAMP oscillations, similar to those observed in other cell types. These rapid cAMP oscillations in somatic (nongerm) cells have been associated with rapid oscillations in intracellular free Ca^{2+} levels. Future studies should examine whether cAMP and Ca^{2+} levels oscillate during progestin-induced induction of hypermotility in fish sperm and the interactions between these two regulators of sperm motility.

Figures

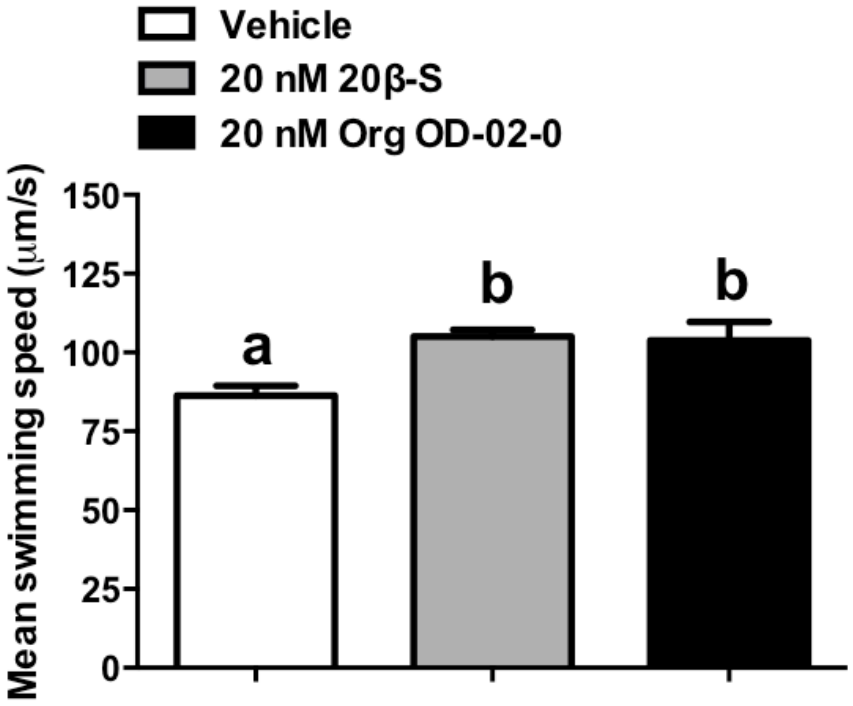


Fig. 2.1. Effects of 1 min treatment with 20β-S and the mPRα agonist (Org OD 02-0) on Atlantic croaker sperm motility. All data represent means ± SEM of the results compiled from 2-3 replicate trials/experiment and from three experiments, $n = 8 – 9$. Similar results were obtained in each of the three experiments. Different letters denote significant differences from each other at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test.

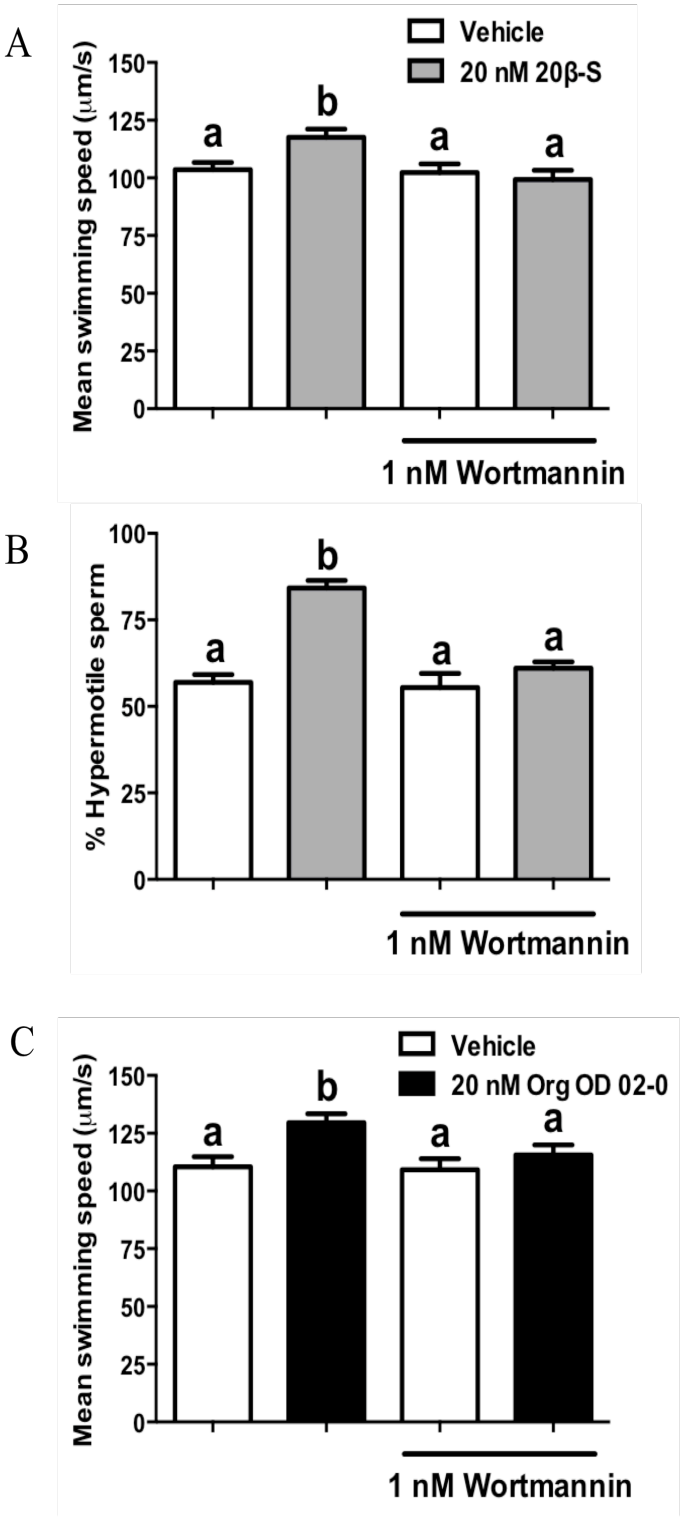


Fig. 2.2. Effects of preincubation with a PI3K inhibitor, Wortmannin (1 nM) on progestin stimulation of Atlantic croaker sperm hypermotility with either 20β-S (A and B) or Org OD 02-0 (C) for 1 min. All data represent means ± SEM of the results compiled from 2-3 replicate trials/experiment and from three to five experiments, n = 8 – 15. The experiment was repeated three times for sperm swimming speed measurements and five times to determine % hypermotile sperm, and similar results were obtained each time. Different letters denote significant differences from each other at P<0.05 level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test.

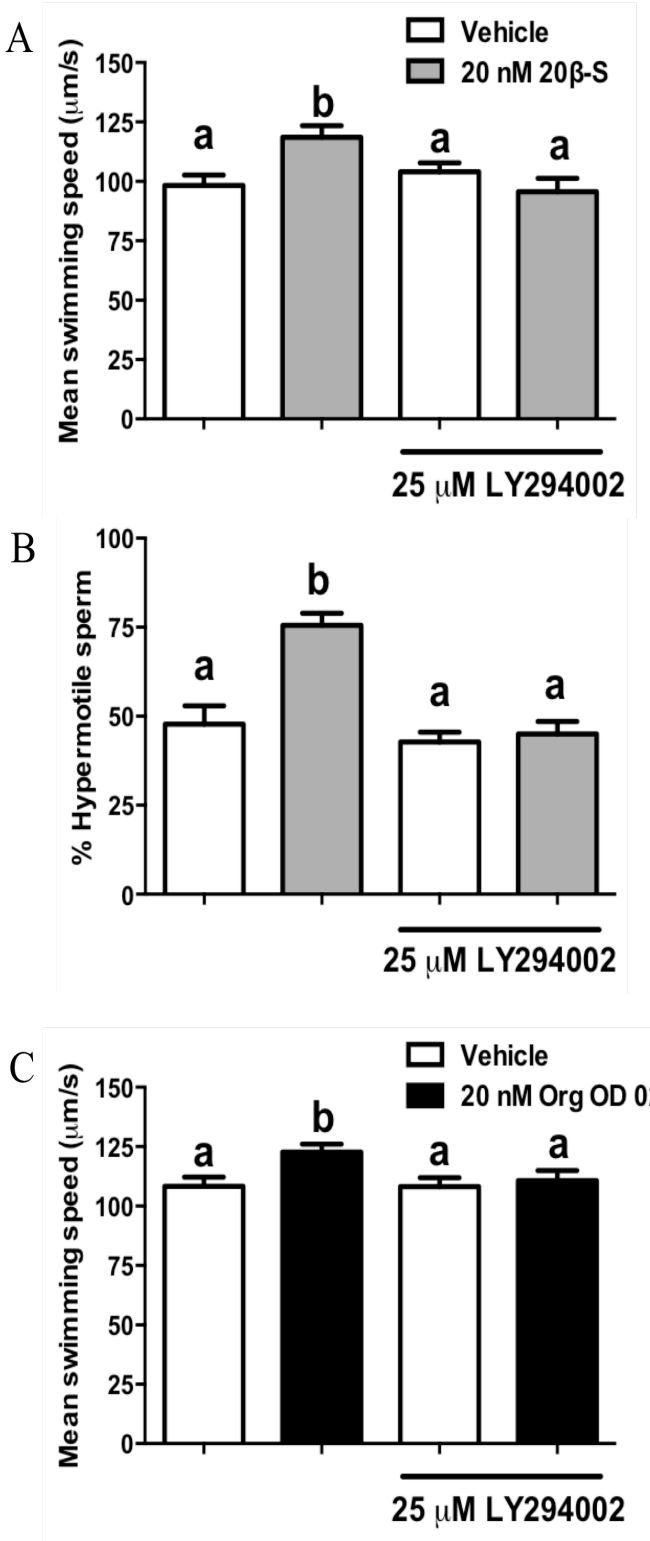
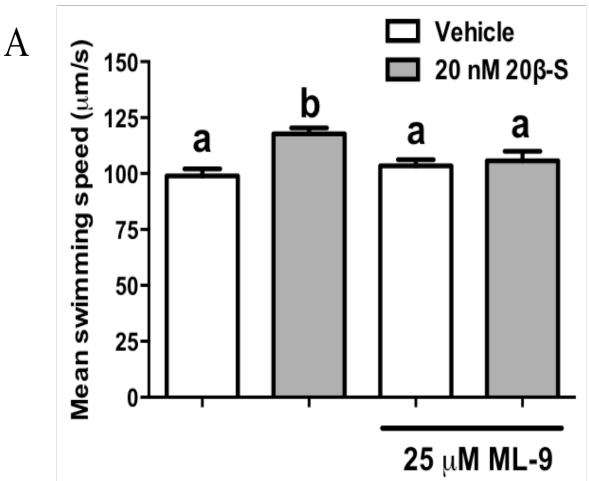
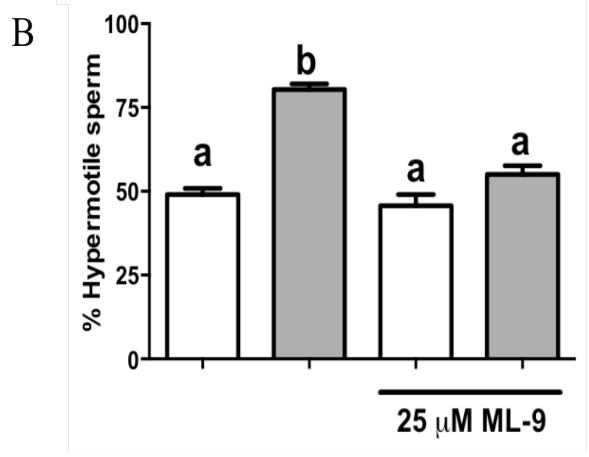


Fig. 2.3. Effects of preincubation with a PI3K inhibitor, LY294002 (25 μM), on progestin stimulation of Atlantic croaker sperm hypermotility with either 20β-S (A and B) or Org OD 02-0 (C) for 1 min. All data represent means ± SEM of the results compiled from 2-3 replicate trials/experiment and from three experiments, n = 8 – 9. The experiment was repeated three times for both sperm swimming speed measurements and determination of % hypermotile sperm, and similar results were obtained each time. Different letters denote significant differences from each other at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test.





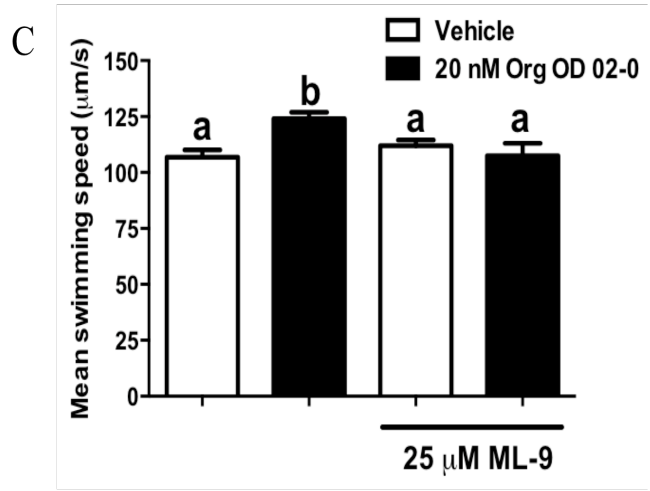


Fig. 2.4. Effects of preincubation with the Akt inhibitor, ML-9 (25 μM), on progestin stimulation of Atlantic croaker sperm motility with either 20β-S (A and B) or Org OD 02-0 (C) for 1 min. All data represent means ± SEM of the results compiled from 2-3 replicate trials/experiment and from three to five experiments, n = 8 – 15. The experiment was repeated three times for sperm swimming speed measurements and five times to determine % hypermotile sperm, and similar results were obtained each time. Different letters denote significant differences from each other at P<0.05 level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test.

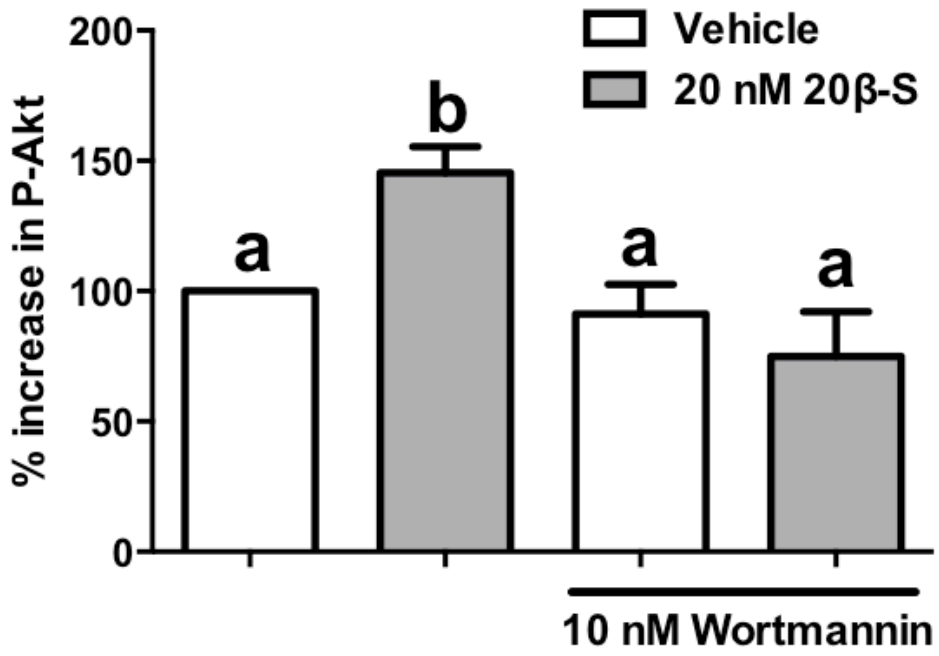


Fig. 2.5. Effects of preincubation with the PI3K inhibitor, Wortmannin (10nM), on Akt phosphorylation of Atlantic croaker sperm in response to 100 nM 20β-S. P-Akt expression was normalized to total Akt on the Western blots and relative densitometries were plotted. All data represent means ± SEM, compiled results from five separate experiments, n = 5. Similar results were obtained in each of the five experiments. Different letters denote significant differences from each other at P<0.05 level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test.

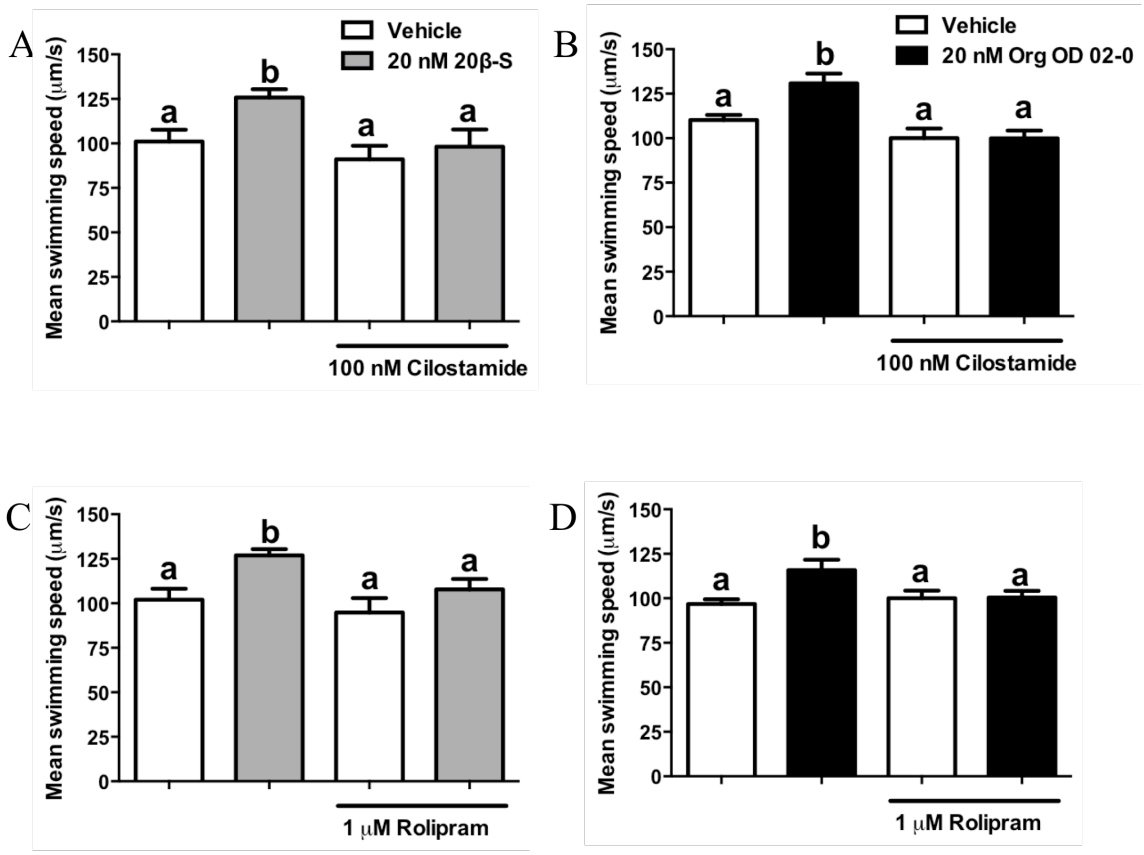
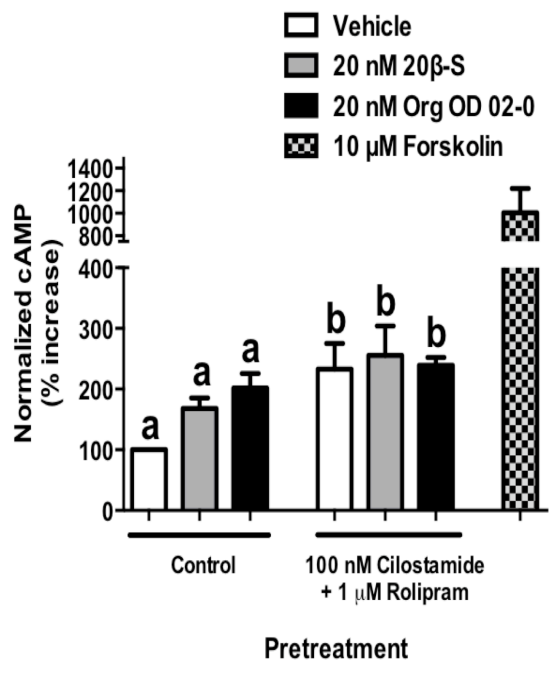


Fig. 2.6. Effects of preincubation with inhibitors of PDE3, Cilostamide (100 nM, A and B) and PDE4, Rolipram (1 μM, C and D) on progestin stimulation of Atlantic croaker sperm hypermotility with either 20β-S (A and C) or Org OD 02-0 (B and D) for 1 min. All data represent means ± SEM of the results compiled from 2-3 replicate samples/experiment and from four experiments, $n = 8 – 11$. Similar results were obtained in each of the four experiments. Different letters denote significant differences from each other at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test.

A



B

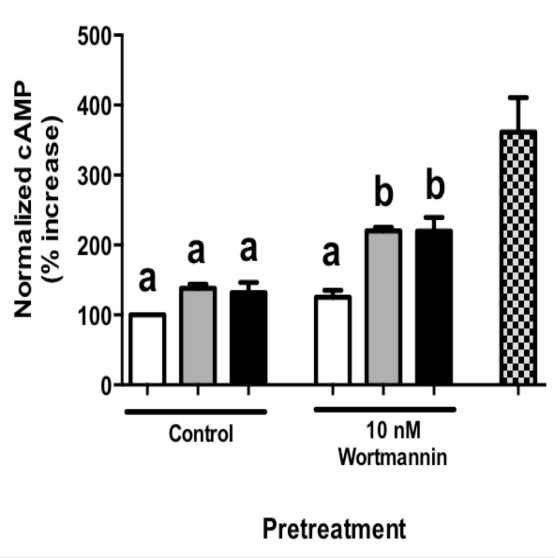


Fig. 2.7. Effects of preincubation of sperm plasma membranes with inhibitors of PDEs (A; 100 nM Cilostamide and 1 μM Rolipram) or PI3K (B; 10 nM Wortmannin) on cAMP production in response to treatment with either vehicle, 20 nM 20β-S, 20 nM Org OD 02-0, or 10 μM forskolin for 1 min. Concentrations of cAMP were normalized to the vehicle-treated control in each experiment to account for donor variability. All data represent means ± SEM of the results compiled from six experiments (1-2 replicates/experiment). Similar results were obtained in each of the six experiments, n = 5 – 9. Different letters denote significant differences from each other at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test.

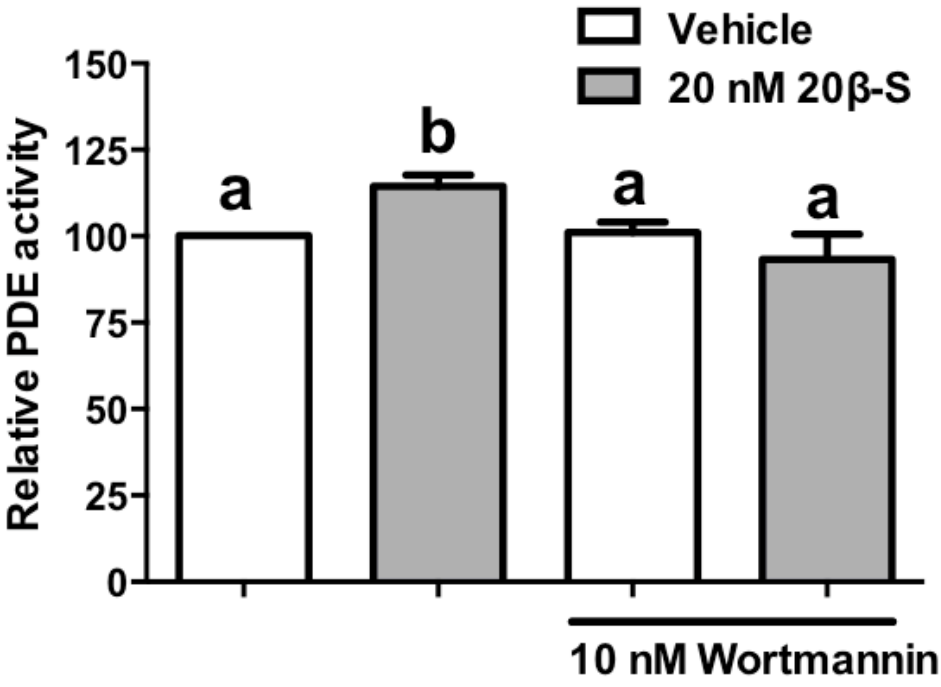


Fig. 2.8. Effects of preincubation of sperm homogenates with an inhibitor of PI3K (10 nM Wortmannin) on progestin stimulation of phosphodiesterase activity with either vehicle or 20 nM for 30 min. Pde activity from each treatment were normalized to vehicle-treated control to account for donor variability. All data represent means ± SEM of the results from seven experiments, $n = 5 – 7$. Similar results were obtained in each of the seven experiments. Different letters denote significant differences from each other at $P<0.05$ level. Significance was determined by one-way ANOVA and Tukey's multiple comparison post-test.

Chapter 3: The epidermal growth factor receptor 2 (ErbB2)/ mitogen-activated protein kinase (Mapk) pathway plays an important role in mediating progestin-induced sperm hypermotility in Atlantic croaker via membrane progestin receptor-alpha

Abstract

Rapid nongenomic stimulation of sperm motility has been observed in multiple vertebrate taxa but the mechanisms governing these effects are poorly understood. In the present study, we investigated whether progestin-stimulation of sperm hypermotility in a teleost, Atlantic croaker (*Micropogonias undulatus*), through membrane progestin receptor-alpha (mPRα, also known as Paqr7b) involves activation of the ErbB2/Mapk pathway. The effects of coincubation with inhibitors of the epidermal growth factor receptor 2 (ErbB2)/ mitogen-activated protein kinase (Mapk) pathway on the induction of croaker sperm motility in response to progestins were tested. Inhibition of upstream regulators of ErbB2, intracellular tyrosine kinase (Src) with PP2 (10 μM) and matrix metalloproteinase (MMP) with Ilomastat (10 μM) effectively abolished progestin-initiated sperm hypermotility. Pretreatment with the human epidermal growth factor receptor 2 (ERBB2) inhibitors AG879 (5 nM) and RG13022 (50 μM) also abrogated progestin stimulation of sperm hypermotility. In addition, preincubation with the human mitogen-activated protein kinase (MAPK) kinase (MEK1/2) inhibitor U0126 (500 nM)

also abolished progestin-stimulated sperm hypermotility. The presence of both extracellular-related kinase 1 and 2 (Erk1/2) and its activated phosphorylated form, P-Erk, in croaker sperm plasma membranes was confirmed by Western blotting. These results demonstrate that progestin-mediated hypermotility via mPRα in Atlantic croaker sperm involves activation of the ErbB2/Mapk pathway.

Introduction

Rapid nongenomic progestin actions to induce sperm hypermotility have been described in several vertebrate species including fish (Thomas, 2003; Tubbs and Thomas, 2009; Tubbs et al., 2011; Chapter 2) and mammals (Uhler et al., 1992; Park et al., 2011). Despite the importance of progestin signaling in regulating sperm physiology and fertility in vertebrates, the mechanisms through which progestins exert these effects remain poorly understood (Thomas et al., 2009; Park et al., 2011). Sperm hypermotility involves increases in the rate and amplitude of flagellar movements resulting in increased velocity and non-linear swimming (Ho and Suarez, 2001). Exposure of Atlantic croaker (*Micropogonias undulatus*) and southern flounder (*Paralichthys lethostigma*) sperm to their endogenous progestin hormone, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) stimulates sperm hypermotility in these species (Thomas, 2003; Thomas et al., 2004; Thomas et al., 2006; Thomas et al., 2011) which has been shown to increase fertilization success (Tubbs and Thomas, 2009; Chapter 4).

Recent evidence suggests that rapid progestin stimulation of sperm hypermotility in several teleost species is mediated through the novel 7-transmembrane membrane progestin receptor alpha (mPRα also known as Paqr7b) (Thomas, 2003; Tubbs and Thomas, 2009, Tubbs et al., 2011), which belongs to the progestin and adipoQ receptor (PAQR) family (Thomas et al., 2007). The mPRα was discovered in spotted seatrout ovaries and shown to be present on the oocyte plasma membrane where it acts as the intermediary in maturation-inducing steroid (MIS) induction of oocyte maturation in this species through a nongenomic mechanism (Zhu et al., 2003). The mPRα protein is also detected on plasma membranes of spotted seatrout sperm (Zhu et al., 2003; Tubbs and Thomas, 2008) as well as on the sperm of the Atlantic croaker, red drum, and southern flounder (Thomas et al., 2006; Tubbs and Thomas, 2009; Tubbs et al., 2011). Several lines of evidence suggest that progestin upregulation of sperm motility through mPRα in these species involves upregulation of sperm cAMP concentrations through increased activity of membrane-bound adenylyl cyclase (Acy) and is associated with activation of the alpha subunit of a stimulatory G protein (Thomas, 2003; Tubbs and Thomas, 2009; Tubbs et al., 2011). Co-immunoprecipitation studies have shown that mPRα is closely associated with a stimulatory olfactory G protein (G_{olf}) in Atlantic croaker sperm membranes (Tubbs and Thomas, 2009) and stimulatory G proteins, including G_{olf}, have also been detected on southern flounder sperm membranes (Chapter 3). Moreover, progestin-induced hypermotility of croaker and flounder sperm is associated with rapid increases in cAMP production, both of which are blocked by cotreatment with the Acy inhibitor, dd-Ado, (Tubbs and Thomas, 2009; Tubbs et al., 2011; Chapter 3).

Collectively, these data suggest an important role for cAMP in inducing sperm hypermotility through this G protein α subunit-dependent signaling pathway. However, recent studies demonstrating that progestins can initiate a multiple signaling cascades through mPRα in other vertebrate cells suggest that other signaling pathways may also mediate sperm motility.

The epidermal growth factor receptor/mitogen-activated protein kinase (Egfr/Mapk) pathway is a plausible candidate for an additional signaling mechanism mediating progestin stimulation of sperm hypermotility in teleosts through mPRα. Transactivation of EGFR through activation of stimulatory G protein βγ-subunits has been reported for another steroid membrane receptor, G protein-coupled estrogen receptor-1, (GPER-1) (Filardo et al., 2002). The βγ-subunits of the G proteins activate Src-related kinases, which in turn trigger matrix metalloproteinases (MMP) to cleave pro-heparin-binding epidermal growth factor (pro-HB-EGF). The cleaved pro-HB-EGF can then bind to EGFR, thus activating the receptor and increasing the activities of downstream MAPKs (Filardo and Thomas, 2005). Components of this pathway have been identified in mammalian sperm. Activation of G proteins in bovine sperm has been shown to transactivate the epidermal growth factor receptor (EGFR), which is localized on the head and midpiece, through protein kinase A (PKA) and Src (Etkovitz et al., 2009) and Src has also been detected on the head of human sperm (Lawson et al. 2008). The mPRα protein is localized primarily on the midpiece with lower expression on the flagellum and head of croaker (Tubbs and Thomas, 2009), southern flounder (Tubbs et al., 2011) and human (Thomas et al., 2009) sperm. EGFR has been shown to be involved

in boar sperm motility (Oliva-Hernandez and Perez-Guiterrez, 2008) and a comprehensive proteomic analysis demonstrated that EGF signaling is an important signaling pathway in high fertility bovine sperm (Peddinti et al., 2008). It has been shown in fish models that progestin binding to mPRα can induce extracellular-related kinase 1 and 2 (Erk1/2, also known as Mapk1/3) Mapk phosphorylation in oocytes and ovarian follicle cells (Zhu et al., 2003; Pace and Thomas, 2005; Dressing et al., 2010), presumably through transactivation of an epidermal growth factor receptor. Recently, Peyton and Thomas (2011) showed that inhibition of ErbB2, a member of the EGFR family, partially reduced the ability of zebrafish oocytes to respond to the endogenous MIS, 17α,20β-dihydroxy-4-pregnen-3-one (DHP) which has previously been demonstrated to act through mPRα in this species (Zhu et al., 2003). Interestingly, the mPRα protein has a similar localization to that of EGFR on sperm with a high concentration on the midpiece and some expression also on the flagellum and head of human (Thomas et al., 2009), croaker (Tubbs and Thomas, 2009), and southern flounder (Tubbs et al., 2011) sperm. As mPRα also mediates the stimulatory effects of progestins on Atlantic croaker sperm motility, the possible role of ErbB2 and Erk1/2 pathways in progestin-stimulated sperm hypermotility in Atlantic croaker merits investigation.

In the present study, we tested the hypothesis that progestin-stimulated sperm hypermotility in Atlantic croaker through mPRα is also mediated by transactivation of ErbB2 and activation of Mapk. The effects of upstream inhibitors of ErbB2 transactivation and inhibitors of Mapk activation on induction of croaker sperm hypermotility by the endogenous progestin in this species, 17, 20β, 21-trihydroxy-4-

pregnen-3-one (20β-S), and the specific mPRα agonist, Org OD 02-0 (Kelder et al. 2010) were investigated.

Materials and Methods

Chemicals

The Atlantic croaker progestin hormone, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) was purchased from Steraloids (Newport, RI). The synthetic progestin, 10-ethenyl-19-norprogesterone (Org OD 02-0) was a gift from Organon (Oss, the Netherlands). The Erk and phospho-Erk (P-Erk) antibodies were purchased from Cell Signaling Technology (Danvers, MA). All inhibitors were purchased from Enzo Life Sciences (Farmington, NY). All other chemicals and reagents were purchased from Sigma unless otherwise noted.

Animals

Adult Atlantic croaker were purchased in the fall from local bait shops. Fish were acclimated to the laboratory for two months before use in 12000 L recirculating tanks at 22 – 24 °C and a photoperiod of 11 hr light, 13 hr dark to promote and maintain gonadal development. All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Milt collection

Milt was collected from fully mature male Atlantic croaker as described previously (Detweiler and Thomas, 1998). Briefly, the abdomen of the fish was wiped

dry to prevent exposure to seawater that causes premature sperm activation. Gentle pressure was applied to the abdomen and the expressed milt was collected from the cloaca with a clean syringe and transferred to tubes that were kept on ice. Care was taken to prevent contamination of milt samples with urine, which would also cause premature activation of sperm.

Preparation of sperm plasma membranes

Sperm membranes were isolated as previously described (Thomas et al., 1997) with minor modifications. Briefly, 3 – 5 mL of milt were diluted in 10 mL of ice-cold homogenization buffer [HAED; 25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA (pH 7.6)] and centrifuged at 1000 × *g* to isolate sperm from seminal fluid. Sperm were resuspended in 10 mL HAED with protease inhibitors (Merck, Darmstadt, Germany). Sperm suspensions were forced through a 23.5 gauge needle twice and sonicated at medium power for 6 s on ice. Samples were then centrifuged at 500 × *g* for 10 min at 4 °C to remove the nuclear fraction. The supernatant was transferred to a clean tube and centrifuged at 17000 × *g* for 20 min at 4 °C to pellet the cell membrane fraction. Isolated sperm membranes were used immediately or stored at -80 °C. Protein concentrations of membrane preparations were determined using a Bradford protein assay (Bio-Rad, Hercules, CA).

Sperm motility analyses

Sperm motility experiments were performed as described previously (Detweiler and Thomas, 1998) with minor modifications. Briefly, croaker milt was diluted 200-fold and preincubated in physiological saline with steroid (20β-S or Org OD 02-0) or vehicle

(EtOH, 1%) for 1 min at room temperature. A 2.5 μL aliquot of each sperm suspension was added to 25 μL activator solution on a microscope slide. A coverslip was placed on the slide and sperm motility was recorded for 1 min using a dark field microscope connected to a computer capable of recording high quality videos. Each experiment was recorded using a charge-coupled device camera (Cohu Electronics, San Diego, CA) and digital recording software (Pinnacle, Mountain View, CA). Each treatment was conducted in triplicate per experiment. Sperm swimming velocity was determined using CellTrak motion analysis software (Motion Analysis Corp., Santa Rosa, CA). The mean swimming speed of each replicate was determined and used for graphical analyses.

For experiments with inhibitors, milt was diluted in physiological saline as described above and preincubated with inhibitors of the ErbB2 pathway or vehicle (dimethylsulfoxide, DMSO; 1 %) for 30 min before treatment with steroid or vehicle for 1 min and activation with activator. The effect of inhibition of Src kinase on Atlantic croaker sperm motility was tested by preincubating sperm with 10 μM of the selective Src kinase inhibitor, PP2 (4-amino-5-[4-chloro-phenyl]-7-[t-butyl]pyrazolo[3,4-d]pyrimidine). The effect of a specific inhibitor of MMP on sperm motility was investigated by preincubating sperm with 10 μM Ilomastat. These concentrations of PP2 and Ilomastat have previously been shown to be effective in blocking the Egfr pathway in fish oocytes (Peyton and Thomas, 2011). The effect of inhibitors of ERBB2 on sperm motility was investigated by preincubating sperm with 5 nM AG879 or 50 μM RG13022. The effect of a specific MEK1/2 inhibitor, U0126, was also tested on Atlantic croaker sperm motility at a concentration of 500 nM. Lower concentrations of AG879 and U0126

were used in this study than those used in previous studies (Filardo et al., 2002; Peyton and Thomas, 2011) because these inhibitors were toxic to sperm at higher concentrations resulting in impaired motility. Sperm motility was recorded and analyzed as described above.

Western blot analyses

Approximately 10 μg of membrane protein was added to loading buffer [0.5 M Tris-HCl, 10% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue, 10% glycerol] and resolved on 10% SDS-PAGE gels. After transfer to nitrocellulose membranes, membranes were blocked in a solution containing 5% nonfat milk, 0.1% Tween-20 in PBS [136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4 (pH 7.4)]. Membranes were rinsed with PBS and incubated overnight at 4 °C with primary antibodies directed towards P-Erk or total Erk (Cell Signaling, Boston, MA; 1:1000) in a blocking solution containing 5% BSA and 0.1% Tween-20 in PBS. Membranes were rinsed with PBS and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam, Cambridge, UK) in blocking solution containing 5% nonfat milk and 0.1% Tween-20 in PBS for 1 hr. Proteins were then visualized using SuperSignal West-Pico chemiluminescent substrate (Thermo Fisher Scientific Inc. Rockford, IL) and exposed to X-ray film (GE Healthcare, Buckinghamshire, UK).

Statistical analyses

For all experiments, data are presented as means ± SEM. Statistical significance was determined using one-way ANOVA and Dunnett's multiple comparison post-tests using GraphPad Prism 5 Software (San Diego, CA).

Results

Effects of PP2, a selective inhibitor of Src on progestin-induced sperm hypermotility

As shown previously, both 20β-S (Fig. 3.1A) and Org OD 02-0 (Fig. 3.1B) significantly increased sperm swimming speed, compared to their respective vehicle-treated controls (20β-S: 124.0 ± 10.0 μm/s, vehicle: 99.7 ± 17.5 μm/s, $P<0.001$; Org OD 02-0: 124.9 ± 9.0 μm/s, 108.6 ± 11.2 μm/s, $P<0.01$). Preincubation of the same pool of sperm with 10 μM PP2 for 30 min did not significantly alter basal, vehicle-treated motility, whereas PP2 treatment resulted in abrogation of the progestin-induced hypermotility, which was not significantly different from the vehicle-treated controls (Fig. 3.1A, B).

Effects of Ilomastat, a specific inhibitor of MMP, on progestin-induced sperm hypermotility

Both 20β-S (Fig. 3.2A) and Org OD 02-0 (Fig. 3.2B) significantly increased sperm swimming speed compared to their respective vehicle-treated controls (20β-S: 131.5 ± 8.1 μm/s, vehicle: 110.3 ± 16.5 μm/s, $P<0.01$; Org OD -02-0: 124.6 ± 13.3 μm/s, vehicle: 105.5 ± 13.1 μm/s, $P<0.05$). Preincubation of sperm with 10 μM Ilomastat for 30 min did not significantly affect motility of vehicle treated sperm, whereas it blocked the progestin-induced hypermotility, which was not significantly different from the vehicle-treated controls (Fig. 3.2A, B).

Effects of AG879 and RG13022, specific ERBB2 inhibitors on progestin-induced sperm hypermotility

Both 20β-S (Fig. 3.3A) and Org OD 02-0 (Fig. 3.3B) significantly increased sperm swimming speed respectively, compared to their respective vehicle-treated controls (20β-S: 124.0 ± 4.26 μm/s, vehicle: 110.3 ± 4.1 μm/s, $P< 0.05$; Org OD 02-0: 118.3 ± 3.3 μm/s, vehicle: 105.3 ± 8.2 μm/s, $P<0.05$). Preincubation of sperm with 5 nM AG879 for 30 min did not significantly affect basal sperm motility, whereas it resulted in the loss of the progestin-induced hypermotility, which was not significantly different from the vehicle-treated controls (Fig. 3.3A, B).

Similar results were observed in sperm pretreated with RG13022. Both 20β-S (Fig. 3.4A) and Org OD 02-0 (Fig. 3.4B) increased sperm swimming speed compared to their respective vehicle-treated controls (20β-S: 118.2 ± 6.2 μm/s, vehicle: 96.9 ± 6.3 μm/s, $P<0.001$; Org OD-02-0: 117.9 ± 11.6 μm/s, vehicle: 98.5 ± 13.9 μm/s, $P<0.05$). Preincubation of sperm with 50 μM RG13022 for 30 min did not alter basal sperm motility, whereas it abolished the progestin-induced hypermotility, which was not significantly different from the vehicle-treated controls (Fig. 3.4A, B).

Effects of U0126, a specific inhibitor of MEK1/2 on progestin-induced sperm hypermotility

Both 20β-S (Fig. 3.5A) and Org OD 02-0 (Fig. 3.5B) increased sperm swimming speed compared to their respective vehicle-treated controls (20β-S: 133.2 ± 11.4 μm/s, vehicle: 107.6 ± 9.7 μm/s, $P<0.001$; Org OD 02-0: 126.0 ± 12.6 μm/s, vehicle: 101.8 ± 12.8 μm/s, $P<0.01$). Preincubation of sperm with 500 nM U0126 for 30 min did not

affect basal sperm motility, whereas it eliminated progestin-induced hypermotility, which was not significantly different from the vehicle-treated controls (Fig. 3.5A, B).

Presence of Erk1/2 in Atlantic croaker sperm plasma membrane fractions

Western blot analyses indicate that Erk1/2 in its inactivated and activated (phosphorylated Erk1/2, P-Erk) forms are present in the plasma membrane fractions of Atlantic croaker sperm (Fig 3.6). Single bands of approximately the correct size (~40 kDa) were detected and no other immunoreactive bands were detected.

Discussion

In the present study, we clearly demonstrate that progestin-induced sperm hypermotility in Atlantic croaker through mPRα is partly mediated by the ErbB2/Mapk signaling pathway. Our results provide the first evidence for a role for the ErbB2/Mapk pathway in the control of sperm motility in teleost fish. Furthermore, the results also suggest a plausible mechanism through which the βγ-subunits of the G protein coupled to mPRα (G_{olf}) may influence sperm physiology. The present results showing that the stimulatory effect of 20β-S on croaker sperm motility is mimicked by the specific mPR agonist, Org OD 20-0 (Kelder at al., 2010), are consistent with previous experiments with croaker sperm (Chapter 1) and further support a role for mPRα in mediating this rapid progestin effect in this species. Previous studies have shown that progestin stimulation of hypermotility in croaker sperm through mPRα involves activation of Acy and increased production of cAMP (Tubbs and Thomas, 2009) as well as activation of a

Pi3K/Akt/phosphodiesterase (Pde) pathway (Chapter 1). The finding that the ErbB2/Mapk signaling pathway is also involved indicates the presence of a multifaceted intracellular signaling mechanism controlling progestin stimulation of sperm hypermotility in this species. Taken together the results suggest that sperm hypermotility is under precise and complex control by progestins in this perciform species, possibly reflecting its critical importance for male fertility and successful reproduction.

The MAPK signaling pathway has previously been shown to play an important role in regulating human sperm motility (Almog et al., 2008). In addition, EGFR is involved in the regulation of boar sperm motility (Oliva-Hernandez and Perez-Guiterrez, 2008). Previous work has shown that ERK1/2 MAPK can be activated through an EGFR-dependent signaling pathway by estrogens (Filardo et al., 2002; Peyton and Thomas, 2011). Activation of mPRα-dependent signaling involving EGFR and co-localization of EGFR and mPRα has been reported in basal phenotype breast cancer cells (Zuo et al., 2010). While ErbB2, a member of the EGFR family, has been implicated in mediating the effects of the zebrafish MIS, DHP, via mPRα on oocyte maturation (Peyton and Thomas, 2011) there have been no reports on the possible interactions between mPRα and ErbB2 in teleost sperm motility. The finding that ErbB2 inhibitors exert their effects on denuded zebrafish oocytes (Peyton and Thomas, 2011) indicates ErbB2 has a similar localization as mPRα (Zhu et al 2003) on the oocyte plasma membrane. Similarly, EGFR and mPRα are likely co-localized on sperm since both receptor types are present on both the head and midpiece of sperm across several vertebrate taxa (Etkovitz et al., 2009; Tubbs and Thomas, 2009; Tubbs et al., 2011; Thomas et al., 2009). Our results using two

inhibitors of ERBB2, AG879 and RG13022, indicate that the progestin-induced hypermotility of croaker sperm is also mediated via ErbB2 and are consistent with a close association between ErbB2 and mPRα.

While these results suggest ErbB2 is present in Atlantic croaker sperm, information on the interactions between ErbB2 and mPRα is currently lacking. In human breast cancer cells, activation of a stimulatory G protein causes the βγ-subunits of the G protein to activate Src-related kinases that in turn activate EGFR through MMP activity, increasing the activities of downstream MAPKs (Filardo et al., 2002). Activation of this pathway has also been demonstrated in zebrafish oocytes (Peyton and Thomas, 2011). Findings using inhibitors of Src and MMP suggest that a similar signaling pathway is present in Atlantic croaker sperm as both of them significantly attenuated sperm hypermotility. To further confirm the proposed mechanism, croaker sperm were preincubated with U0126, an inhibitor of MEK1/2, a downstream effector of ERBB2 (Filardo et al., 2002; Peyton and Thomas, 2011) and obtained similar results to those with the other inhibitors, suggesting that Mek1/2 is also an important signaling component of the ErbB2/Mapk pathway in progestin-stimulated sperm hypermotility in Atlantic croaker. Finally, the presence of Erk1/2 and its activated form, P-Erk, were also confirmed in the plasma membrane of croaker sperm, indicating that this is a possible effector of the ErbB2/Mapk pathway. The proposed mechanism of the ErbB2/Mapk pathway in progestin-initiated sperm hypermotility in croaker is shown in Fig. 3.7.

Protein kinase A (PKA) is also known as cAMP-dependent protein kinase and its activity is dependent on cellular levels of cAMP (Tasken and Aandahl, 2004). It has been

shown that croaker sperm cAMP levels are increased after acute pretreatment with 20β-S (Thomas, 2003; Tubbs and Thomas, 2009), which presumably results in activation of PKA. Interestingly, Etkovitz et al (2009) previously reported that transactivation of EGFR could be mediated by both Src and PKA. Therefore, PKA may also modulate the transactivation of ErbB2 in croaker sperm. It will be important to test this hypothesis because it would provide a plausible mechanism through which the α-subunit of the G_{olf} protein could interact with the signaling pathways activated by the G protein βγ-subunit upon progestin activation. Moreover, another possible mechanism through which different signaling cascades may interact has previously been described (Chapter 1). It was clearly demonstrated that progestin-stimulated sperm hypermotility in croaker is at least partially mediated by the Pi3k/Akt pathway maintenance of Pde activity; Pde activity is significantly increased with the treatment of 20β-S, which results in a reduction in intrasperm cAMP levels. However, a previous study by Thomas and Tubbs (2009) demonstrated that progestin induction of sperm hypermotility in Atlantic croaker is also dependent upon Acy activation and increased intrasperm cAMP concentrations. Taken together, these findings suggest that progestin-initiated sperm hypermotility through mPRα in teleost sperm involves modulation of intracellular cAMP concentrations through multiple signaling pathways.

While little is known about how the PI3K/AKT pathway may intersect with the ERBB2/MAPK pathway in the context of sperm motility, studies of sperm capacitation and acrosome reaction have shown that the PI3K pathway is downstream of EGFR signaling, and that inhibition of the PI3K pathway with Wortmannin effectively blocked

the acrosome reaction induced by EGF (Etkovitz et al., 2009; Breitbart and Etkovitz, 2011). These results are consistent with observations in ram sperm where capacitation, which includes hyperactivation and precedes the acrosome reaction, is reduced when EGFR, PKA, or MEK are inhibited (Luna et al., 2012). While the acrosome is indeed absent in teleost sperm, it should be noted that the same signaling components found in mammalian sperm are also present in teleost sperm. Therefore, it would be interesting to explore the potential interactions between the Pi3k/Akt and ErbB2/Mapk pathways in progestin stimulation of sperm motility in teleosts.

In conclusion, the results of the present study clearly show that progestin-induced sperm hypermotility in Atlantic croaker mediated through mPRα involves the ErbB2/Mapk pathway. Taken together with previous studies of croaker sperm (see also Chapter 1), our findings highlight the complexity of the mechanisms that govern progestin-induced sperm hypermotility. Future studies should examine whether progestin-induced hypermotility involves the activation of Erk1/2, and the possible intracellular crosstalk between the α-subunit (Acy/cAMP pathway)) and the βγ-subunits (Pi3k/Akt/Pde and ErbB2/Mapk pathways) of the G protein associated with mPRα (G_{olf}).

Figures

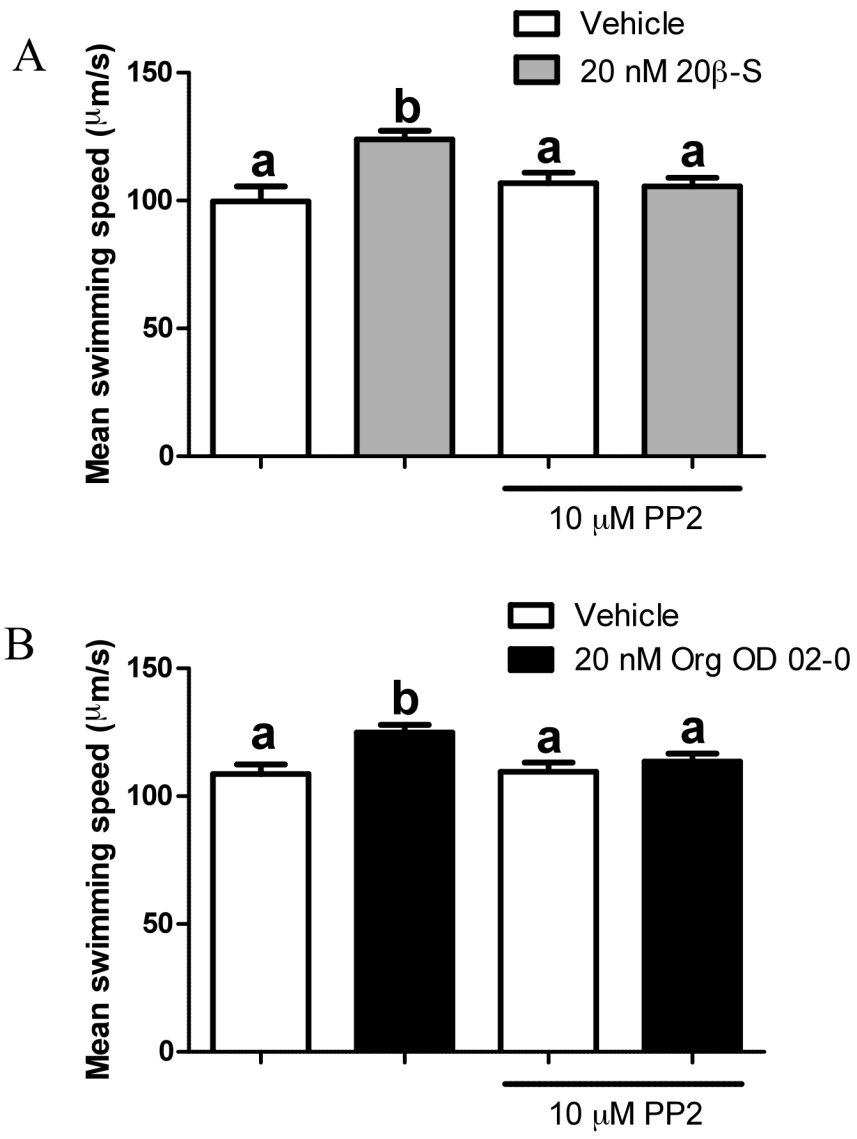


Fig. 3.1. Effects of preincubation with the Src inhibitor, PP2 (10 μM), on progestin stimulation of Atlantic croaker sperm motility with either 20β-S (A) or Org OD 02-0 (B) for 1 min. All data represent mean ± SEM, *n* = 9. Shared letters denote no significant differences at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test. The experiment was repeated three times and each treatment was conducted in triplicate and similar results were obtained each time.

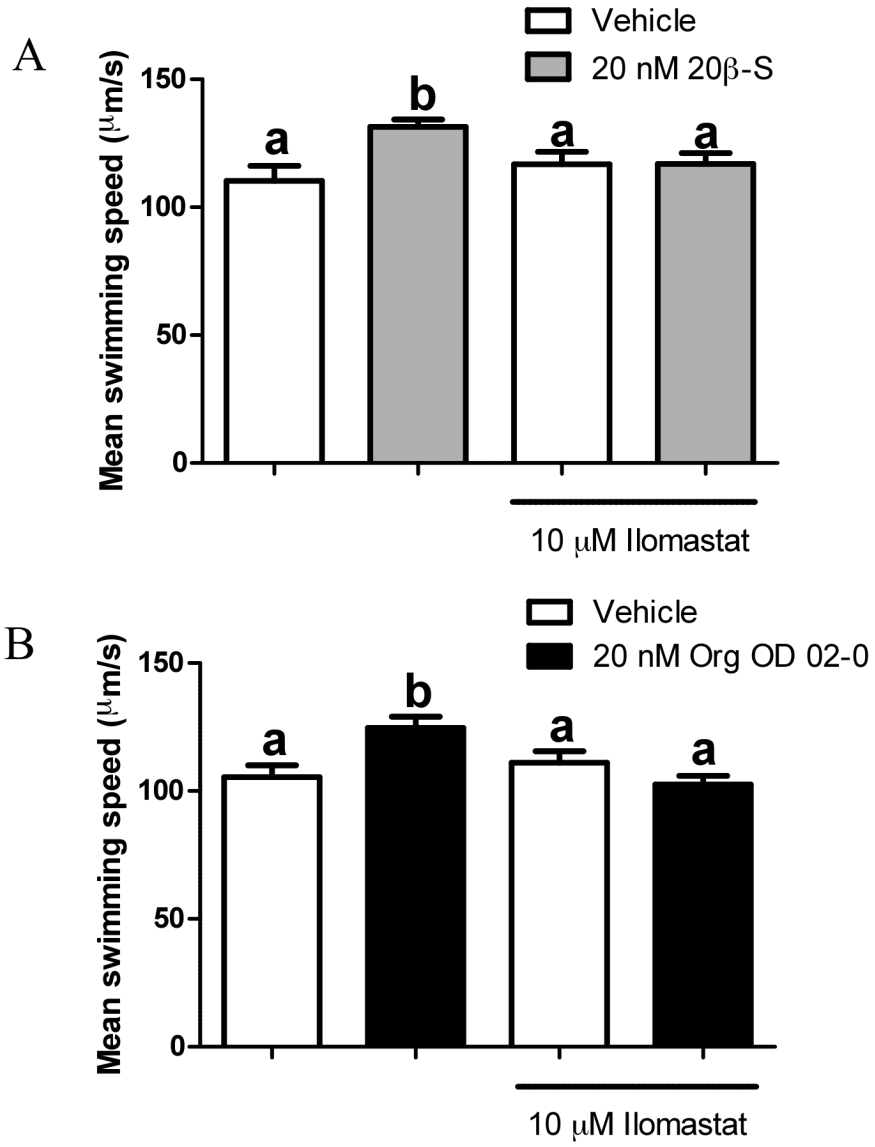


Fig. 3.2. Effects of preincubation with the MMP inhibitor, Ilomastat (10 μM), on progestin stimulation of Atlantic croaker sperm motility with either 20β-S (A) or Org OD 02-0 (B) for 1 min. All data represent mean ± SEM, n = 8 – 9. Shared letters denote no significant differences at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test. The experiment was repeated three times and each treatment was conducted in triplicate and similar results were obtained each time.

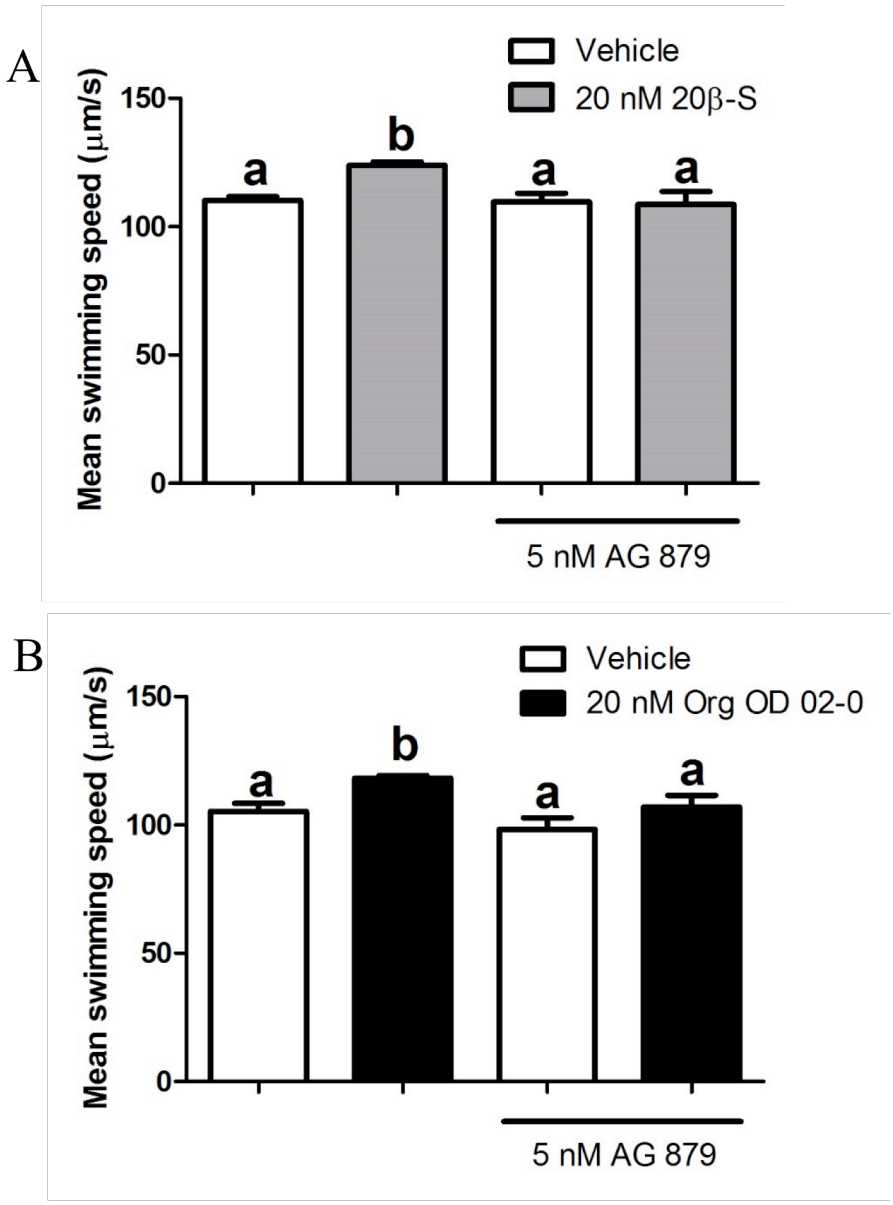


Fig. 3.3. Effects of preincubation with the ERBB2 inhibitor, AG879 (5 nM), on progestin stimulation of Atlantic croaker sperm motility with either 20β-S (A) or Org OD 02-0 (B) for 1 min. All data represent mean ± SEM, n = 8 – 12. Shared letters denote no significant differences at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test. The experiment was repeated four times and each treatment was conducted in triplicate and similar results were obtained each time.

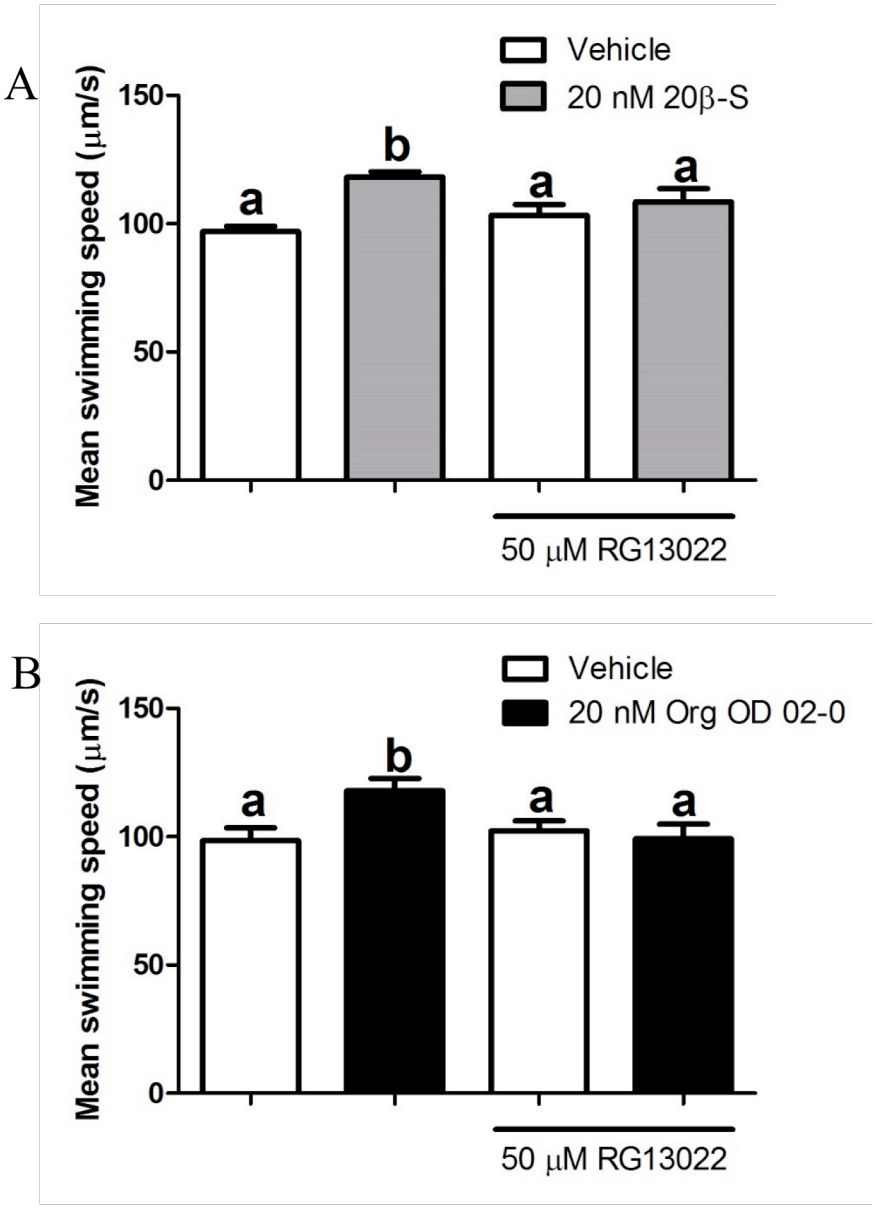


Fig. 3.4. Effects of preincubation with the ERBB2 inhibitor, RG13022 (50 μM), on progestin stimulation of Atlantic croaker sperm motility with either 20β-S (A) or Org OD 02-0 (B) for 1 min. All data represent mean ± SEM, n = 8 – 9. Shared letters denote no significant differences at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test. The experiment was repeated three times and each treatment was conducted in triplicate and similar results were obtained each time.

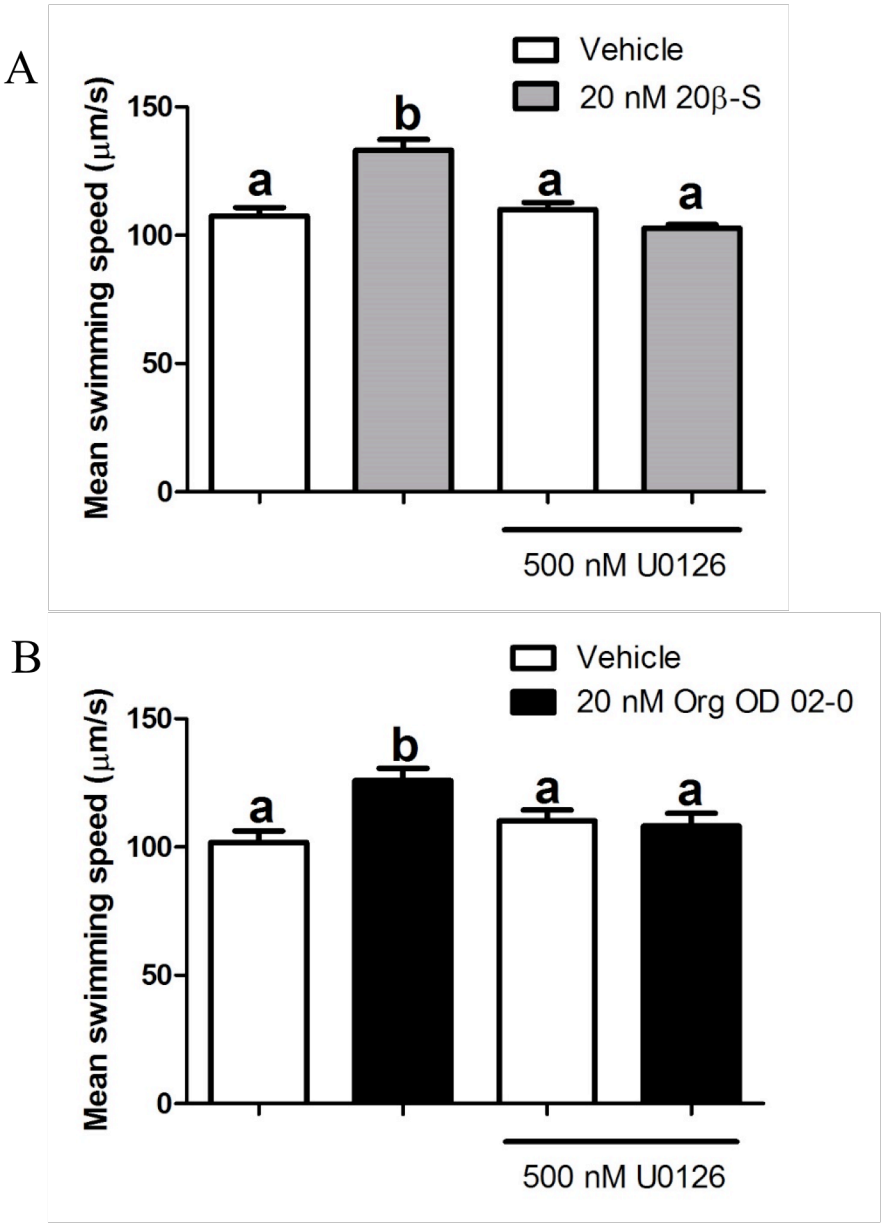


Fig. 3.5. Effects of preincubation with the MEK1/2 inhibitor, U0126 (500 nM), on progestin stimulation of Atlantic croaker sperm motility with either 20β-S (A) or Org OD 02-0 (B) for 1 min. All data represent mean ± SEM, n = 8 – 9. Shared letters denote no significant differences at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test. The experiment was repeated three times and each treatment was conducted in triplicate and similar results were obtained each time.

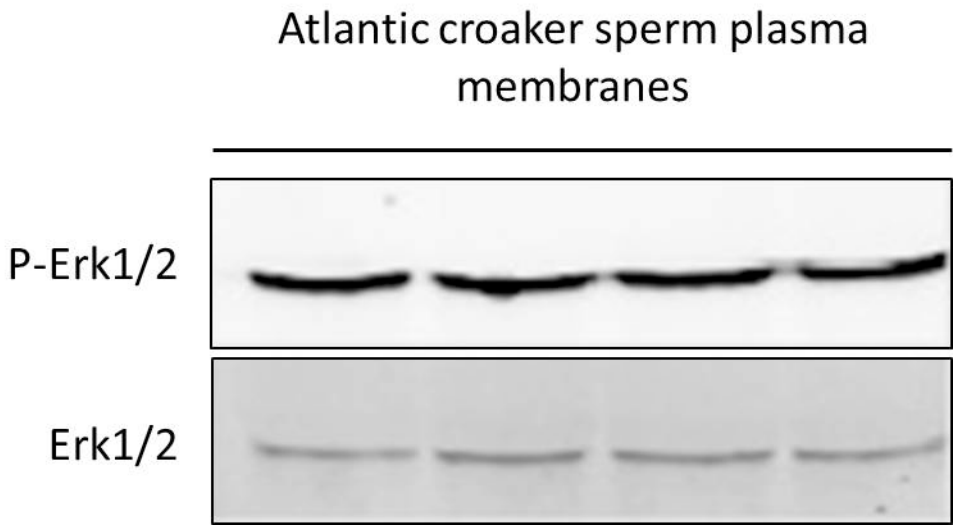


Fig. 3.6. Western blot analyses indicate that Erk and its activated form, P-Erk are present on Atlantic croaker sperm plasma membranes. The experiment was repeated three times and similar results were obtained each time.

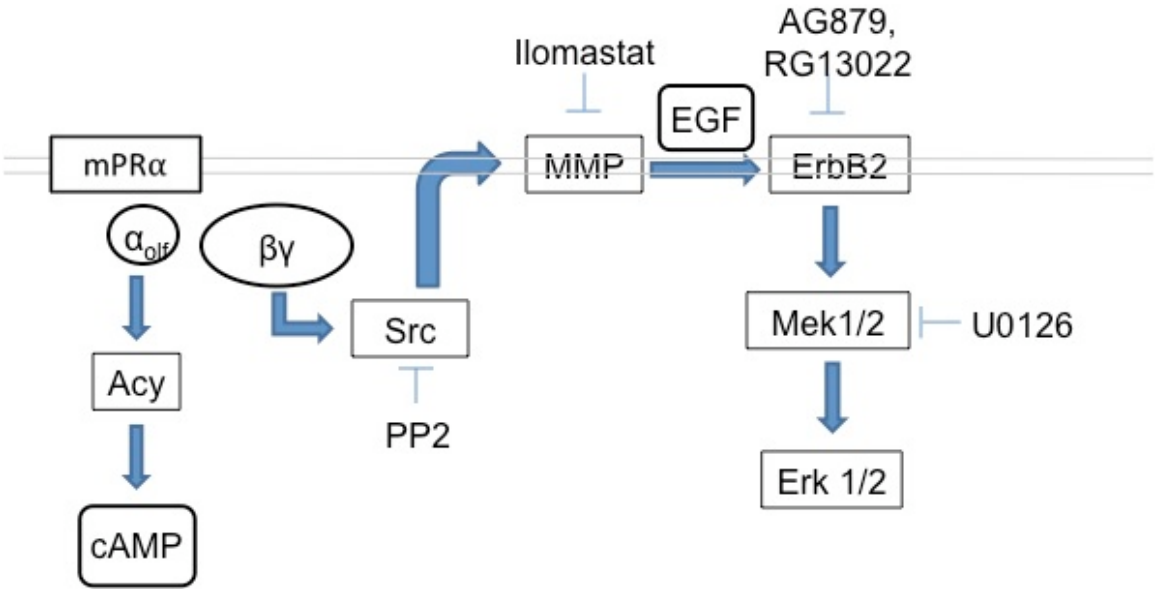


Fig. 3.7. Proposed model of progestin signaling through mPRα to stimulate sperm hypermotility in Atlantic croaker. The sites of action of the inhibitors used in the present study are also shown. Progestin binding to mPRα results in activation of a G_{olf}. The G protein βγ-subunit transactivates Egfr through Src and MMP, which results in the phosphorylation of Erk1/2 to stimulate sperm hypermotility.

Chapter 4: Membrane progestin receptor-alpha mediates progestin-induced sperm hypermotility and increased fertilization success in southern flounder (*Paralichthys lethostigma*).

Abstract

Progestin hormones stimulate sperm motility in teleosts but their mechanisms of action remain unclear. Preliminary results suggest that progestin upregulation of sperm motility in southern flounder and several other marine species is mediated through a sperm membrane progestin receptor with the characteristics of membrane progestin receptor-alpha (mPRα, also known as Paqr7b). The hypothesis that mPRα has an important role in progestin regulation of southern flounder sperm motility and fertility was tested in the present study. The *mPRα* mRNA was shown to be highly expressed in several tissues, including the brain, olfactory epithelium and testes. Immunocytochemical analyses indicated that the mPRα protein was localized to the sperm plasma membrane and concentrated on the sperm midpiece, with weak expression on the flagella. Sperm from high motility donors had higher concentrations of mPRα on their plasma membranes than sperm from low motility donors. The specific mPRα agonist, 10-ethenyl-19-norprogesterone (Org OD 02-0, 100 nM), mimicked the stimulatory actions of the endogenous progestin, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S, 100 nM) on flounder sperm motility. The expression of mPRα protein on the sperm plasma membranes was positively correlated to sperm motility as well as the responsiveness of

sperm to progestin stimulation. Acute *in vitro* progestin treatment of sperm with high mPRα protein expression increased both sperm motility and fertilization success in strip spawning experiments, but was ineffective on sperm with low receptor expression. A single injection of the superactive GnRH analog (LHRHa, 100μg/kg) increased sperm motility and fertilization success in strip spawning experiments 72 hr post-injection, which was accompanied by an increase in mPRα protein expression on sperm plasma membranes. These results provide clear evidence that southern flounder sperm hypermotility and fertility are mediated through mPRα. Preincubation of flounder sperm with the specific membrane adenylyl cyclase (Acy) inhibitor, dd-Ado, abrogated progestin-stimulated sperm hypermotility. Stimulatory G proteins (G_s and G_{olf}), but not inhibitory G proteins (G_i), were identified in flounder sperm plasma membrane fractions. The finding that treatment of flounder sperm plasma membrane fractions with either 20β-S or Org OD 02-0 increases cAMP levels suggests progestins stimulate flounder sperm motility by activating a stimulatory G protein/Acy pathway. A similar mechanism has been identified in Atlantic croaker, suggesting that the signaling pathway mediated by mPRα in sperm is highly conserved in advanced teleosts. Collectively, our results indicate that progestin-stimulation of flounder sperm hypermotility and fertility are dependent on sufficient mPRα expression which can be upregulated by *in vivo* LHRHa treatments. These findings potentially have practical applications for enhancing the fertility of male flounder broodstock.

Introduction

Progestins have been shown to rapidly induce sperm hypermotility in a variety of vertebrate species, but the membrane receptors and signaling pathways mediating this nongenomic progestin action remain equivocal and controversial. Recently, it was shown that *in vitro* treatment with the major progestin produced by southern flounder (*Paralichthys lethostigma*) testes, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), induces sperm hypermotility in this species, whereas other teleost steroid hormones and the nuclear progestin agonist, R5020, were ineffective (Tubbs et al., 2011). A high affinity, specific 20β-S receptor was characterized on flounder sperm membranes and is the likely mediator of 20β-S induction of sperm hypermotility. The receptor appears to be coupled to a G protein because [^{3}H]-20β-S binding to flounder sperm membranes was decreased after treatment with guanosine 5'-O-[gamma-thio]triphosphate ($GTP_\gamma S$) which dissociates G proteins from their receptors (Tubbs et al., 2011). Finally, preliminary evidence was obtained that the 20β-S receptor is membrane progestin receptor-alpha (mPRα, also known as Paqr7b) as mPRα was cloned from flounder testes and the abundance of the mPRα protein on the sperm plasma membrane fraction was found to be positively correlated to flounder sperm motility (Tubbs et al., 2011), similar to findings in Atlantic croaker (Tubbs and Thomas, 2009) and human sperm (Thomas et al., 2009).

Although these results suggest that mPRα mediates progestin stimulation of sperm hypermotility in southern flounder, loss-of-function experiments using siRNA to block mPRα transcription to confirm this cannot be conducted because mature vertebrate sperm are transcriptionally inactive. However, the recent discovery of a specific mPR

agonist, 10-ethenyl-19-norprogesterone (Org OD 02-0) that does not activate the nuclear progesterone receptor (PR) (Kelder et al., 2010), provides a valuable tool for determining the role of mPRα in mediating vertebrate sperm hypermotility.. Therefore, Org OD 02-0 was used extensively in the present study to verify that the progestin-induced sperm hypermotility and fertilization success is mediated through mPRα.

Rapid progestin stimulation of sperm hypermotility has also been demonstrated in three members of the Sciaenidae family, spotted seatrout (*Cynoscion nebulosus*), Atlantic croaker (*Micropogonias undulatus*), and red drum (*Sciaenops ocellatus*) (Thomas 2003; Thomas et al., 2006; Tubbs and Thomas, 2008). The proposed mechanism involves binding of progestins to a receptor on the sperm plasma membrane that in turn causes rapid increases in intracellular cyclic adenosine monophosphate (cAMP) and Ca^{2+} levels resulting in sperm hypermotility (Thomas 2003; Thomas et al., 1997). The discovery that mPRα is coupled to a stimulatory olfactory G protein (G_{olf}) in Atlantic croaker sperm corroborates the cAMP results because activation of the G_{olf} α-subunit by progestins would be expected to increase the activity of membrane-bound adenylyl cyclase (Acy) (Tubbs and Thomas, 2009). Furthermore, treatment of croaker sperm with the Acy inhibitors, dd-Ado or SQ22536, was shown to block progestin-induced hypermotility and the increase in cAMP levels (Tubbs and Thomas, 2009). Similarly, treatment of flounder sperm with dd-Ado attenuated the response to progestins, indicating that progestin-stimulated sperm motility is also dependent on Acy activity in flounder sperm (Tubbs et al., 2011). However, changes in cAMP levels in southern flounder sperm in response to progestin stimulation have not been reported.

The biological significance of the progestin-induced increase in flounder sperm motility has not been determined. For example, it has not been demonstrated that the increased flounder sperm swimming velocity influences male fertility. Similarly, there have been no reports on the effects of hormonal manipulation of male southern flounder on the quality of the milt produced and on fertilization success. Previous experiments with Atlantic croaker showed that sperm swimming speeds and fertility were increased by an injection of a superactive gonadotropin-releasing hormone analog (LHRHa; des-Gly10,[d-Ala6]LHRH 91–90 ethylamide) and were associated with an increase in mPRα protein expression on the sperm plasma membrane (Tubbs and Thomas, 2009). In view of the commercial importance of southern flounder and its potential as an aquaculture species, hormonal manipulation of male flounder to increase fertilization success may be a practical method to improve the reproductive efficiency of captive broodstock.

In the present study, we tested the hypothesis that progestin-stimulated sperm hypermotility in southern flounder is mediated through mPRα and that sperm with higher swimming velocities have higher fertilization success compared to that of lower motility sperm. We also investigated whether higher sperm motility and the ability to respond to progestins were associated with the increased concentrations of mPRα on sperm plasma membranes. Finally, we also examined whether hormonal treatments could produce higher motility flounder sperm with higher fertilization success.

Materials and Methods

Chemicals

LHRH analog (LHRHa; des-Gly^{10},[d-Ala^{6}]LHRH (1–9) ethylamide) was purchased from Bachem (Torrance, CA). The progestin, 17,20,21-trihydroxy-4-pregnen-3-one (20β-S) was purchased from Steraloids (Newport, RI). The synthetic progestin, 10-ethenyl-19-norprogesterone (Org OD 02-0) was a gift from Organon (Oss, the Netherlands). 2',5'-dideoxyadenosine (dd-Ado) was purchased from Calbiochem (La Jolla, CA). The cAMP assay kit was purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals and reagents were purchased from Sigma unless otherwise noted.

Animals

Adult southern flounder were captured with nets in the Corpus Christi Ship Channel near Port Aransas, TX during their reproductive season (October-November) and immediately transferred to the wet lab facilities at the University of Texas Marine Science Institute. Flounder were maintained in 35 ppt salinity seawater in 12,000 L recirculating tanks at 18 °C with a photoperiod of 11L:13D and fed shrimp daily to promote and maintain gonadal development. Fish were acclimated to laboratory conditions for at least 4 weeks prior to use in experiments. All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Expression of membrane progestin receptor mRNA and protein

Total RNA from flounder brain, olfactory epithelium, gill, heart, liver, muscle, testis, stomach, kidney, and intestine was prepared as previously described (Tubbs et al.,

2010). One-step qRT-PCR was performed using Brilliant II SYBR Green qRT-PCR Master Mix Kit, 1-step (Agilent Technologies, La Jolla, CA) following the manufacturer's instructions with 5 pmol of the following primers: *mPRα* sense 5'-GCTGCCTTCATCATCTTGGT-3', antisense 5'-CGCTGAAGGAGAGGTAGGTG-3'; 18S rRNA sense 5'-AGAAACGGCTACCACATCCA-3', antisense 5'-TCCCGAGATCCAACTACGAG-3'. Expression of *mPRα* relative to flounder 18S rRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Immunolocalization of mPRα on flounder sperm was performed as described previously with minor modifications (Tubbs and Thomas, 2009). The concentrations of the spotted seatrout primary and the AlexaFluor 488 secondary antibody used were 1:1000 and 1:2000, respectively. Sperm were visualized using Nikon C1 confocal and Eclipse E600 microscopes.

Milt collection

Milt was collected from fully mature male southern flounder as described previously (Tubbs et al., 2011). Briefly, the abdomen of the fish was wiped dry to prevent exposure to saltwater that causes premature sperm activation. Gentle pressure was applied to the abdomen and the expressed milt was collected from the cloaca with a clean Pasteur pipette and samples were kept on ice. Care was taken to prevent contamination of milt samples with urine, which would also cause premature activation of sperm. Past experience showed that sperm displaying higher motility generally came from donors that milted in large volumes (~ 1 mL) and that produced opaque white milt.

Sperm motility analyses

Milt was collected and sperm motility experiments were performed as described previously (Detweiler and Thomas, 1998; Thomas and Doughty, 2004) with minor modifications. Milt from 2-4 flounder was pooled according to their observed traits (e.g. volume and color) diluted 50-fold in physiological saline (osmolarity 340 mOs/kg, pH 7.8) and preincubated with steroid (20β-S or Org OD 02-0) or vehicle (EtOH, 1%) for 1 min at room temperature. A 2.5 μL aliquot of each sperm suspension was added to 25 μL activating solution (osmolarity 680 mOs/kg, pH 7.8) on a microscope slide and sperm motility of all motile sperm in the field of view was recorded for 1 min using a dark field microscope connected to a computer capable of recording high quality videos. Each experiment was recorded using a charge-coupled device camera (Cohu Electronics, San Diego, CA) and digital recording software (Pinnacle, Mountain View, CA). Sperm swimming speed (average path velocity) was determined using CellTrak motion analysis software (Motion Analysis Corp., Santa Rosa, CA). Each treatment was conducted in triplicate per experiment and each experiment was repeated six times (progestin spawning trials) or seven times (LHRHa injection trials). Data presented represent the means of the average swimming speeds ± SEM.

The role of Acy in progestin stimulation of sperm motility was investigated using the Acy inhibitor, dd-Ado. Sperm were preincubated with dd-Ado (50 μM) or vehicle (0.01% DMSO) for 20 min in physiological solution before carrying out sperm motility analyses as described above.

Spawning experiments

Progestin *in vitro* treatment of flounder sperm

Experiments on the effects of progestins on southern flounder sperm motility and fertility were conducted in October and November in 2011. Six trials were conducted. Female flounder were selected based on their reproductive state; only females that showed significant ovarian development (visible ovarian swelling) were used. Females were injected intramuscularly with 100 μg/kg of LHRHa approx. 72 h before spawning trials to initiate final oocyte maturation. Eggs were stripped by applying gentle pressure to the abdomen of the fish. Eggs were collected in a beaker and mixed with saltwater. Only the floating mature eggs were used in spawning experiments. Eggs from a single female donor were used for each spawning trial.

Milt was collected from 2-4 males as described above. Pools of high and low motility milt were diluted in physiological saline 10X, divided among the different treatment groups, and treated with either vehicle, 100 nM 20β-S, or 100 nM Org OD 02-0 for 5 min before addition to beakers containing ~ 3 mL eggs in 100 mL of clean seawater to final concentrations of milt of 1:10,000 or 1:20,000 by volume. Eggs and sperm were stirred for 10 s with a feather and fertilization was allowed to proceed for 30 min before the addition of 500 mL seawater. Fertilization success was determined by counting the number of fertilized eggs (eggs undergoing cell division 2-3 h post fertilization) per treatment by random sampling of eggs in quadruplicate and expressing the result as a percentage (% fertilization = number of fertilized eggs/total number of eggs × 100).

LHRHa *in vivo* treatment of male flounder

Experiments to examine the effects of LHRHa treatment on male sperm motility and fertility were conducted in December 2012, towards the end of the flounder

reproductive season to determine if the functional characteristics and mPRα expression of relatively low motility and fertility sperm could be improved by hormonal treatment of donors with LHRHa. Forty milting males were randomly divided into control and treatment groups. Four spawning trials were conducted with 10 males per trial. Treatment males were given a single intramuscular injection of LHRHa (100 μg/kg) while control males were given a single injection of saline vehicle (0.9% NaCl). Milt was collected from two males at 0 h post-injection. Milt was collected from two males at 0 h post-injection, and from two males per treatment group at times 24 and 72 h post-injection. Sperm motility analyses were conducted on sperm from individual donors as described above. Three additional sperm motility assays were conducted in the absence of viable females. Milt was frozen for Western analyses. Milt collected at 72 h post-injection was also used in spawning experiments, where pools of milt from treatment and control groups were tested for their ability to fertilize eggs following the protocol described above. Females were injected with LHRHa to induce ovulation of mature eggs. Pools of milt from donors either injected with 100 μg/kg LHRHa or saline were diluted 10X in physiological saline, divided among the different treatment groups, and incubated for 5 min with either vehicle, 100 nM 20β-S, or 100 nM Org OD 02-0 before addition to beakers containing ~ 3 mL eggs in 100 mL of clean seawater to final concentrations of milt of 1:10,000 or 1:20,000 by volume. Fertilization was induced following procedures described previously and fertilization success was calculated as outlined above. Twenty fertilized eggs were randomly selected and placed into glass finger bowls and allowed to

proceed to hatching which was expressed as percent hatch (% hatch = number of hatched offspring/total number of eggs × 100).

Preparation of sperm plasma membranes

Sperm membranes were isolated as previously described (Thomas et al., 1997) with minor modifications. Briefly, 3 – 5 mL of milt were diluted in 10 mL of cold homogenization buffer [HAED; 25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA (pH 7.6)] and centrifuged at 1000 × *g* to isolate sperm from seminal fluid. Sperm were resuspended in 10 mL HAED with protease inhibitors (Merck, Darmstadt, Germany). Sperm suspensions were forced through a 23.5 gauge needle twice and sonicated at medium power for 6 s on ice. Samples were then centrifuged at 500 × *g* for 10 min at 4 °C to remove the nuclear fraction. The supernatant was transferred to a clean tube and centrifuged at 17000 × *g* for 20 min at 4 °C to pellet the cell membrane fraction. Isolated sperm membranes were analyzed immediately. Protein concentrations of membrane preparations were determined using a Bradford protein assay (Bio-Rad, Hercules, CA).

Western blot analyses

Approximately 10 µg of membrane protein was added to loading buffer [0.5 M Tris-HCl, 10% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue, 10% glycerol] and resolved on 10% SDS-PAGE gels. After transfer to nitrocellulose membranes, membranes were blocked in a solution containing 5% nonfat milk, 0.1% Tween 20 in PBS [136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4 (pH 7.4)]. Membranes were rinsed with PBS and incubated overnight at 4 °C with primary

antibodies directed against a 15-mer peptide sequence in the N-terminal region of spotted seatrout mPRα (Paqr7b) (YRQPDQSWRYYFLTL; 1:2000), which differs from the corresponding flounder peptide sequence at only one position (underlined) and has previously been validated for detection of mPRα in southern flounder sperm (Tubbs et al., 2011). A pan-actin antibody (Novus Biologicals, Littleton, CO) was used to confirm equal protein loading. Membranes were rinsed with PBS and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam, Cambridge, UK) in blocking solution containing 5% nonfat milk and 0.1% Tween 20 in PBS for 1 h. Proteins were then visualized using SuperSignal West-Pico chemiluminescent substrate (Thermo Fisher Scientific Inc. Rockford, IL). Densitometry analyses of the bands were performed using ImageJ and data were normalized to the 0 h samples to account for inter-trial variability on separate Western blots. Expression of G proteins was determined using antibodies directed against G_s, G_{olf} or G_i (Santa Cruz Biotechnology, Dallas, TX).

Measurement of cAMP production by croaker sperm membranes

Sperm cell membranes were resuspended in buffer [75 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EDTA (pH 7.6)] to a total protein concentration of 1 mg/mL. To investigate the effects of the Acy inhibitor, dd-Ado, on cAMP levels, sperm cell membrane preparations were preincubated with 50 μM dd-Ado or vehicle for 30 min. Membrane preparations (100 μL) were added to assay buffer [0.2 mM ATP, 10 nM GTP, 0.5 mM phosphoenolpyruvate, 20 μg pyruvate kinase, 2 mM 3-isobutyl-1-methylxanthine (IBMX)] containing 20 nM 20β-S, 50 nM Org OD 02-0, 10 μM forskolin

(Acy activator) or vehicle. Reactions were allowed to proceed for 1 min at room temperature and then samples were boiled for 5 min and centrifuged at 14,000 × *g* for 10 min. Supernatants were collected, diluted 10- to 20-fold, and cAMP concentrations were determined using a commercial cAMP enzyme immunoassay kit following the manufacturer's instructions (Cayman Chemincal, Ann Arbor, MI). Data were normalized to mean control values to account for donor variability.

Statistical analyses

For all experiments, data are presented as means ± SEM. Statistical significance was determined using a Student's *t* test or one-way ANOVA and Dunnett's or Tukey's multiple comparison post-tests using GraphPad Prism Software (San Diego, CA).

Results

Expression of membrane progestin receptor mRNA and protein in flounder tissues

Quantitative RT-PCR analyses demonstrate that *mPRα* mRNA is most highly expressed in flounder brain, olfactory epithelium, testis, gill and heart. Weak expression of *mPRα* mRNA was detected in stomach, kidney, liver, intestine and muscle (Fig. 4.1).

Immunocytochemical analyses demonstrate that the mPRα protein is localized to the midpiece of flounder sperm. No staining was evident on sperm heads and weak staining for the receptor was present on the sperm flagella (Fig. 4.2).

Association between mPRα abundance and sperm velocity

Flounder producing low quantities of milt at the end of the reproductive season in

2009 had a mean sperm velocity (72.2 ± 4.2 μm/s) that was not significantly different to that of fish producing greater quantities of milt during this period (89.2 ± 8.1 μm/s) (Fig. 4.3B). Treatment with 20 nM 20β-S significantly increased mean sperm velocity in the higher motility group ~30% to 121.1 ± 11.0 μm/s (P<0.05, Fig. 4.3B), whereas the velocity was not significantly increased by 20β-S treatment in the low motility group (Fig. 4.3B). Abundance of mPRα was approximately 2.9-fold greater on sperm membranes from the higher sperm velocity donor group than on membranes from the lower velocity group as determined by Western blot analyses (Fig. 4.3A). To further investigate the association between mPRα abundance and differences in baseline sperm motility, milt was collected at the peak of the reproductive season in 2010, when sperm was of overall better quality (i.e., higher motility). There was no apparent difference in milt volumes from fish producing low motility or high motility sperm. However, high motility sperm displayed significantly higher swimming velocity (105.0 ± 2.8 μm/s; P<0.05, Fig. 4.3B) compared to low motility sperm (mean velocity = 80.3 ± 4.7 μm/s, Fig. 4.3B). Western blot analysis showed that the higher motility sperm had a 2.3-fold greater abundance of mPRα than lower motility sperm (Fig 4.3A).

***In vitro* progestin upregulation of flounder sperm motility depends on initial motility**

Sperm from high volume and opaque milt samples showed higher basal sperm motility (90.1 ± 6.6 μm/s) compared to sperm from low volume and dilute male samples (61.3 ± 5.8 μm/s; P<0.05, Fig. 4.4A). The velocity of high motility sperm was significantly increased after treatment with either 100 nM of the endogenous progestin, 20β-S (120.2 ± 6.3 μm/s; P<0.05, Fig. 4.4A), or the selective mPRα agonist, Org OD 02-

0 (118.8 ± 5.4 μm/s; $P<0.05$, Fig. 4.4A). Low motility sperm did not show a significant change in their swimming speeds when treated with either 100 nM of 20β-S or Org OD 02-0 (Fig. 4.4A).

Treatment of high motility sperm with progestins increases fertilization success

The same pools of milt from the sperm motility experiments were used in strip spawning experiments to test the hypotheses that faster swimming sperm have better fertilization success rates and that *in vitro* progestin treatment of sperm increases fertilization success. At the higher milt concentration of 1:10,000, high motility sperm showed higher fertilization success (23.2 ± 1.5 %) compared to low motility sperm (7.1 ± 1.9 %; $P<0.001$, Fig. 4.4B). At the lower milt concentration of 1:20,000, high motility sperm still showed higher fertilization success (15.30 ± 1.23 %) compared to low motility sperm (1.42 ± 0.55 %; $P<0.001$, Fig. 4.4C).

As previously shown (Fig. 4.4A), high motility sperm responded to treatment of either 20β-S or Org OD 02-0 and increased their swimming speed. Treatment of sperm with either 20β-S or Org OD 02-0 also increased the fertilization success at both 1:10,000 (34.9 ± 2.4 % ($P<0.001$) and 34.5 ± 2.8 % ($P<0.001$), respectively; Fig. 4.4B) and 1:20,000 dilutions (22.9 ± 1.6 % ($P<0.05$) and 25.4 ± 3.0 % ($P<0.001$), respectively; Fig. 4.4C). The motility of low motility sperm was previously shown to be unresponsive to both progestin treatments (Fig. 4.4A) and neither progestin treatment had any significant effect on fertilization success of low motility sperm (Fig. 4.4B, C).

***In vitro* progestin upregulation of flounder sperm motility and fertilization success depends on elevated sperm expression of mPRα**

Plasma membranes were extracted from both high and low motility sperm. Western blot analyses indicated that high motility sperm samples contained higher protein concentrations of mPRα compared to low motility sperm samples; with relative mPRα abundances of 1.5 ± 0.2 and 0.7 ± 0.2, respectively (P<0.05, Fig. 4.5A, B).

Injection of LHRHa increases flounder sperm motility

Southern flounder males were injected with 100 µg/kg of LHRHa or saline and milt was collected at 0, 24, and 72 h post-injection. Sperm collected from donors at 0 h showed a slight but significant increase in swimming speed when treated with 20β-S (100.3 ± 2.6 µm/s; P<0.05) compared to the vehicle treated controls (87.4 ± 3.2 µm/s, Fig. 4.6). Sperm collected at 72 h post-injection from males injected with a single 100 µg/kg dose of LHRHa showed higher (108.8 ± 3.0 µm/s; P<0.05) sperm motility than the controls at 0 h (Fig. 4.6). Sperm collected at this time point did not show any further stimulation of swimming speed when treated with either 100 nM 20β-S (109.3 ± 3.0 µm/s) or Org OD 02-0 (107.1 ± 2.4 µm/s, Fig. 4.6). Sperm collected from donors injected with saline did not show any significant increases to sperm motility at 24 h or 72 h post-injection when treated with either 20β-S or Org OD 02-0 (Fig. 4.6).

Injection of LHRHa increases flounder fertilization success

Milt collected from males 72 h post-LHRHa injection showed significant increases in fertilization success at both 1:10,000 (35.8 ± 4.2 %; P<0.001, Fig. 4.7A) and 1:20,000 (22.5 ± 2.5 %; P<0.001, Fig. 4.7B) dilutions compared to fertilization success at these dilutions of sperm collected from donors injected with saline (5.8 ± 2.1 % and 0.5 ± 0.3 %) (Fig. 4.7A, B). Similar to the observation that motility of sperm from LHRHa-

treated donors could not be further stimulated by addition of exogenous 20β-S or Org OD 02-0 treatments (Fig. 4.6), addition of these progestins also did not significantly increase fertilization success over the high levels achieved with LHRHa injection alone (Fig. 4.7A, B). Sperm motility of donors that were injected with saline vehicle were shown to be unresponsive to progestin stimulation (Fig. 4.7A, B) and the addition of either 20β-S or Org OD 02-0 did not significantly affect fertilization success at either 1:10,000 or 1:20,000 dilutions (Fig. 4.7A, B). Embryos fertilized with sperm that had been treated with either 20β-S or Org OD 02-0 did not show any adverse developmental defects and the hatching rate was comparable between treatment groups and control at over 95% hatch (data not shown). Only eggs fertilized by sperm from LHRHa-treated males were used for this experiment as fertilization rates from vehicle-injected donors were very low (Fig. 4.7B).

LHRHa-injected males produced sperm with higher mPRα concentrations

Western blot analysis of sperm samples from LHRHa-injected males at 72 h post-injection showed they had significantly higher mPRα protein concentrations (287.8 ± 93.9 %) in the plasma membrane fraction compared to those of 0 h post-injection samples (100%) and vehicle-injected samples at 72h post-injection. (Fig. 4.8A, B) Sperm from vehicle-injected fish did not show any significant alteration in the mPRα expression (Fig. 4.8A, B).

Effects of membrane adenylyl cyclase inhibitor on sperm motility

Pre-incubation of flounder sperm with a cell-permeable Acy inhibitor, dd-Ado (50 μM) was effective in blocking the induction of sperm hypermotility by 20 nM 20β-S

(mean velocity = 88.0 ± 3.1 μm/s) when compared to sperm pre-incubated in vehicle (0.01% DMSO) and treated with 20 nM 20β-S (mean velocity = 119.8 ± 5.1 μm/s; $P<0.05$, Fig. 4.9). Pre-incubation with dd-Ado did not affect basal motility of flounder sperm as there was no significant difference between the mean velocity of sperm pre-incubated with vehicle (mean velocity = 91.6 ± 3.2 μm/s) and sperm pre-incubated with dd-Ado (mean velocity = 94.8 ± 2.9 μm/s, Fig. 4.9).

Flounder sperm expresses only stimulatory G proteins

Western blot analyses of flounder sperm plasma membranes indicated that while the stimulatory G proteins (G_s and G_{olf}) could be detected, no immunoreactive bands were detected for inhibitory G proteins (G_i) (Fig. 4.10A).

Flounder sperm hypermotility is correlated to increased internal cAMP levels

Cyclic AMP levels were significantly increased in flounder sperm after treatment with 20β-S (179.4 ± 20.5 % of vehicle treated controls), Org OD 02-0 (186.1 ± 39.4 % of vehicle treated controls), or a selective Acy agonist, forskolin (199.3 ± 21.7 % of vehicle treated controls, Fig. 4.10B). We have previously shown that preincubating flounder sperm with the Acy inhibitor, dd-Ado, abolished 20β-S-stimulated sperm hypermotility (Fig. 4.9, Tubbs et al., 2011). Preincubation of sperm membranes with dd-Ado also blocked the production of cAMP (Fig. 4.10B), suggesting that a rise in internal cAMP levels is required for progestin stimulation of sperm hypermotility in this species.

Discussion

The results of the present study provide compelling evidence that progestin regulation of sperm motility in southern flounder is mediated through mPRα. Although previous studies have indicated that mPRα is involved in mediating 20β-S-induced sperm hypermotility in this species (Tubbs et al., 2011), direct evidence was lacking because loss-of-function experiments with siRNA to knockdown mPRα expression cannot be performed in sperm which is transcriptionally inactive. However, the recent discovery of a selective mPRα agonist, Org OD 02-0, that does not activate the nuclear progesterone receptor (Kelder et al., 2010), provides a valuable alternative tool for investigating mPRα functions in sperm. The present results showing that Org OD 02-0 mimics the stimulatory actions of the endogenous southern flounder progestin, 20β-S, in stimulating sperm hypermotility, provide convincing evidence that mPRα is the receptor mediating progestin upregulation of sperm motility in this species and also corroborates our previous findings. The observations that these progestin treatments also increase cAMP production, an action blocked by co-treatment with an Acy inhibitor, dd-Ado, and that stimulatory G proteins are present on flounder sperm membranes, further support our earlier proposed mechanism of progestin stimulation of sperm motility in this species through a mPRα/stimulatory G protein/Acy/cAMP signaling pathway (Tubbs et al., 2011). In addition, several lines of evidence clearly indicate that high sperm motility and the stimulatory actions of progestins are dependent on sufficient expression of mPRa on flounder sperm membranes. Low motility sperm with low levels of mPRα expression were unresponsive to progestins, whereas higher motility sperm had higher membrane mPRα concentrations and showed a further increase in sperm motility in response to 20β-

S and Org OD 02-0 treatments. Moreover, LHRHa-induced upregulation of sperm motility was accompanied by increased mPRα levels compared to those in saline injected controls. A second major finding of the current study is that these increases in sperm mPRα expression and motility are correlated with marked increases in the fertilization success of sperm in strip spawning experiments. Collectively, these results demonstrate that mPRα plays a critical role in the maintaining the motility and fertility of southern flounder sperm.

Sperm hypermotility is a major determinant of male fertility in fish and other vertebrates because it is required for fertilization of eggs (Auger et al., 1994; Gasparini et al., 2010). Sperm hypermotility involves an increased rate and amplitude of sperm flagella movements leading to increased velocity and non-linear swimming, which have been shown to increase oocyte contact and fertilization (Ho and Suarez, 2001; Tubbs and Thomas, 2009; Gasparini et al., 2010). Progestin stimulation of sperm motility has been reported in a variety of mammalian and teleost species (Uhler et al., 1992; Thomas, 2003; Tubbs and Thomas, 2009; Park et al., 2011) including southern flounder (Tubbs et al., 2011), but prior to this study it was not known if progestin stimulation of sperm hypermotility in southern flounder translates to increased fertilization success. The present results clearly show that incubation of flounder sperm with either 20β-S or the selective mPRα agonist, Org OD 02-0 induced hypermotility and significantly increased fertilization success of sperm pooled from high motility donors, which had high plasma membrane expression of the mPRα protein. In contrast, sperm from low motility donors with lower concentrations of mPRα were unresponsive to the progestin treatments. The

association between high motility of flounder sperm and increased mPRα expression has been reported previously (Tubbs et al., 2011). The current results provide the first clear evidence that high mPRα expression is also essential for high fertility of vertebrate sperm.

Currently, we have limited knowledge of the intracellular signaling mechanisms mediating progestin-induced sperm hypermotility in southern flounder through mPRα. Coimmunoprecipitation experiments have shown that mPRα is closely associated with a stimulatory G protein, $G_{olf,}$ in another advanced marine teleost belonging to the superorder Acanthopterygii, Atlantic croaker (Tubbs and Thomas, 2009). A model proposed for progestin activation of this stimulatory G protein through mPRα in croaker sperm is supported by the observations that 20β-S treatment increases cAMP production by croaker sperm plasma membranes and that treatment with the Acy inhibitor, dd-Ado, attenuates 20β-S-induced hypermotility (Tubbs and Thomas, 2009). Similar motility experiments conducted with flounder sperm using dd-Ado suggest sperm hypermotility is also at least partially mediated by alterations in intracellular cAMP in this species (Tubbs et al., 2011). Additional support for this mechanism is provided by the present results showing the presence of stimulatory G proteins, G_s and $G_{olf,}$, in flounder sperm plasma membranes as well as progestin-induced increases in sperm cAMP production which were abrogated by dd-Ado treatment. Taken together, these findings suggest that the progestin signal transduction pathway mediating sperm hypermotility through mPRα and Acy is conserved in advanced fishes.

A single injection of LHRHa (100μg/kg body weight) was predicted to cause upregulation of flounder sperm mPRα protein and sperm motility, because this treatment was shown to be effective in increasing these sperm functions in Atlantic croaker (Tubbs and Thomas, 2009). Although LHRHa treatment presumably increases sperm mPRα protein levels through activation of the pituitary-testis reproductive axis, the principal hormone regulating mPRα expression remains unclear. The maturation-inducing steroids 20β-S and 17,20β-dihydroxy-4-pregnen-3-one (DHP) are candidates for the hormones regulating mPRα expression because *in vivo* administration of gonadotropin-releasing hormones such as LHRHa causes increases in gonadal production of these steroids in teleosts with fully recrudesced gonads through their stimulation of luteinizing hormone (LH) secretion (Nagahama, 1997; King and Pankhurst, 2004; Yueh et al., 2005). Moreover, it has been demonstrated that mPRα expression is directly upregulated *in vitro* in seatrout and zebrafish oocytes by 20β-S or DHP, respectively (Zhu et al., 2003; Pang and Thomas, 2010). However, insulin-like growth factor II also directly upregulates mPRα expression in southern flounder oocytes *in vitro* (Picha et al., 2012). Gonadotropin treatments also increase mPRα concentrations and progestin receptor binding in fish oocytes and sperm (Zhu et al., 2003; Tubbs et al., 2010), but their mechanism of action remains equivocal because both steroidogenesis-dependent and -independent actions have been reported (Thomas et al., 2005; Tubbs et al., 2010).

The discovery that acute *in vitro* treatment of southern flounder sperm with progestin hormones can cause marked increases in fertilization success in strip spawning experiments has potential practical applications in aquaculture to improve male fertility

of southern flounder broodstock and possibly other teleost species. Southern flounder is an attractive species for aquaculture due to its desirable aquaculture characteristics; e.g. growth rates, euryhaline tolerance, and high international market value (Smith and Denson, 2000). Although significant progress has been made in spawning this species in captivity by manipulation of environmental conditions (Arnold et al., 1977; Watanabe et al., 2001) and hormonal treatments (Berlinsky et al., 1996), relatively low fertilization rates have often been reported (Watanabe et al., 2001; Smith et al., 1999). Inadequate male reproductive performance, sperm quality and milt production are considered major causes of the relatively poor fertilization and reproductive success of southern flounder spawning in captivity (Henderson-Arzapalo et al., 1988; Smith and Denson, 2000). For example, in one study sperm from only 11 of 25 broodstock males were motile after activation in seawater (Berlinsky et al., 1996). Whereas all the male donors in the current study produced motile sperm, in approximately 40% of them motility was low, with velocities of only ~ 60 μm/s, compared to the average swimming speed of high motility sperm (~ 90 μm/s). These low motility sperm were unresponsive to progestin treatment and exhibited low fertility, resulting in percent fertilization rates of less than 10%, even in spawning trials using higher concentrations of sperm (1:10000 dilution). The results suggest that acute progestin treatment is only effective in increasing motility and fertility of sperm with sufficient mPRα protein levels and motilities above 90 μm/s. The finding that five minutes *in vitro* treatment with the progestins caused approximately a 50% increase in fertility suggests this facile treatment would be a useful addition to strip

spawning procedures for southern flounder and has the potential advantage of not requiring handling of the broodstock and the associated stress prior to milt collection.

Captive fish generally exhibit symptoms of reproductive dysfunction (Zohar and Mylonas, 2001). Thus, hormonal manipulation for inducing reproduction in fish has been used since the 1930s (Crim and Glebe, 1990). Our results showing *in vivo* administration of LHRHa was also effective in increasing of male reproductive performance in southern flounder sperm are consistent with those of previous studies with other flounder species in which increased milt volume and in some cases sperm motility were reported after *in vivo* administration of superactive GnRH peptides (Clearwater, 1998; Shangguan, 1999; Lim et al., 2002, 2004; Moon et al., 2003). Our results suggest that the effectiveness of a single 100μg/kg LHRHa injection in increasing sperm motility and fertility of southern flounder sperm 72 h later is comparable to that achieved by the acute *in vitro* progestin treatments, with both procedures increasing sperm velocity by ~20 – 30 μm/s and fertilization success to ~ 35 % in spawning trials using higher sperm concentrations (1:10000 dilutions). It is important to note that although the LHRHa treatment also increased sperm mPRα expression, additional *in vitro* treatment with progestins failed to further increase sperm motility. One possible interpretation of these findings that requires further evaluation is that the sperm from LHRHa-injected donors had already reached their maximum potential velocity due exposure to elevated progestin levels *in vivo* and therefore were unresponsive to further progestin stimulation *in vitro*.

In conclusion, we provide clear evidence that progestin-stimulation of southern flounder sperm hypermotility is mediated by mPRα and involves activation of an

Acy/cAMP pathway. These results suggest that the signaling pathway mediating progestin upregulation of sperm motility is conserved in advanced fishes. We also provide the first evidence that this progestin–induced increase in sperm motility is of physiological importance because it results in increased fertilization success in strip spawning experiments. Thus, *in vitro* treatment of fish sperm with progestins has potential practical applications in aquaculture for improving the fertility of male broodstock. Southern flounder sperm motility and fertilization success can also be increased by a single injection of LHRHa 72 h prior to fertilization.

Figures

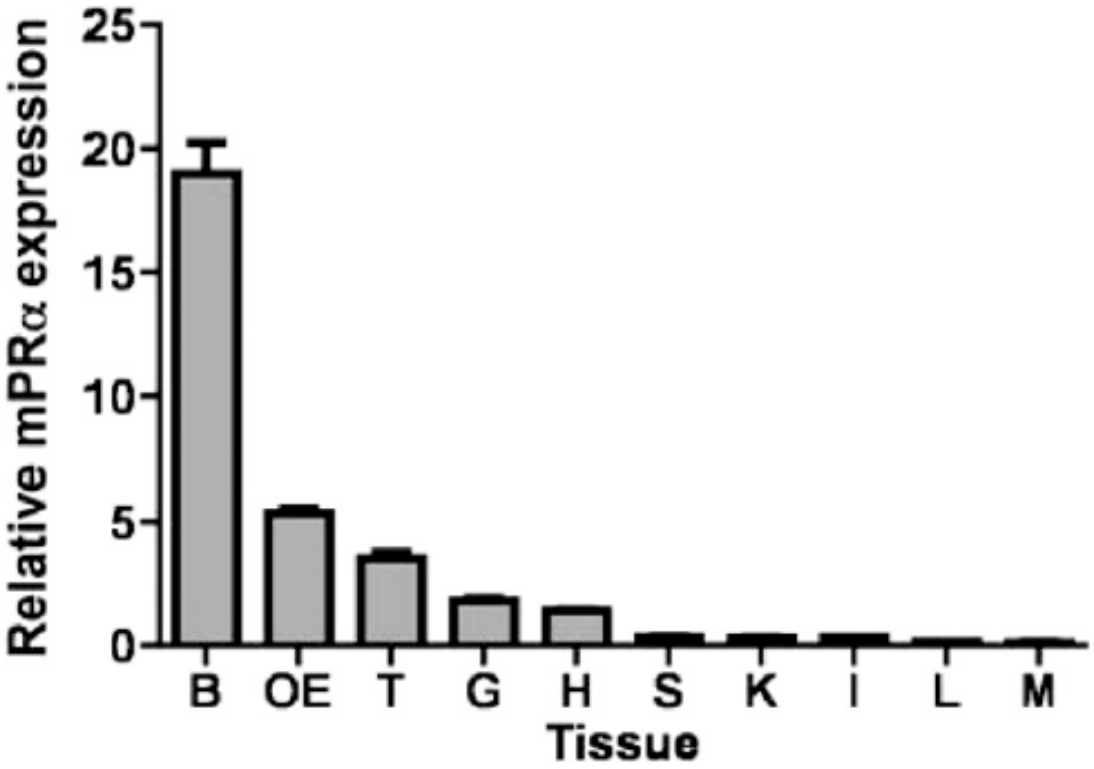


Fig. 4.1. Expression of mPRα mRNA in southern flounder tissues. Quantitative RT-PCR analyses of mPRα mRNA expression relative to 18S rRNA in flounder brain (B), olfactory epithelium (OE), testis (T), gill (G), heart (H), stomach (S), kidney (K), intestine (I), liver (L) and muscle (M). Adapted from Tubbs et al., 2011.

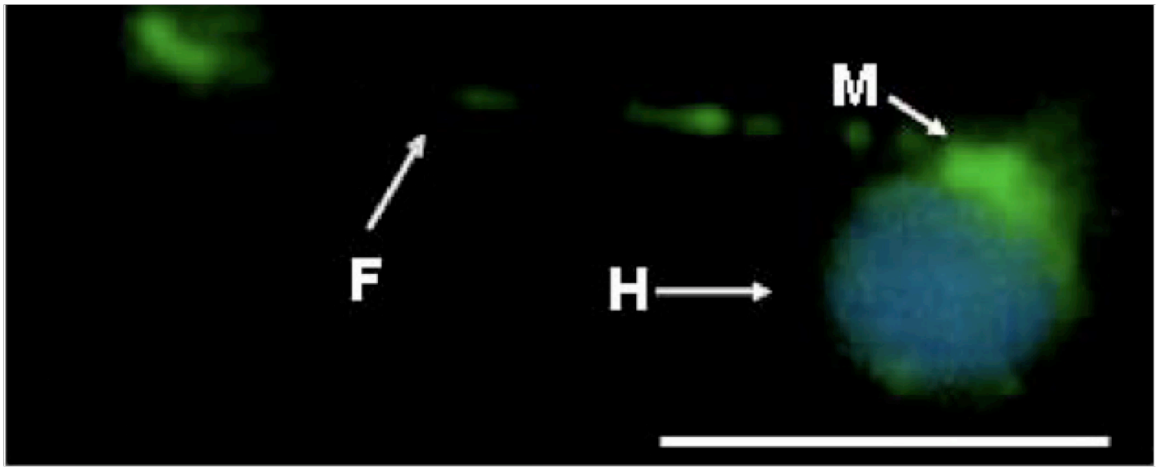


Fig. 4.2. Immunocytochemical analyses of mPRα expression (green) on flounder sperm using the spotted seatrout receptor primary antibody (1:1000) and an AlexaFluor 488 secondary antibody (1:2000). Bar = 5 μm. M, midpiece; F, flagella; and H, head (stained blue with 40,6-diamidino-2-phenylindole, DAPI, 1 mg/ml). Results are typical of 3 independent experiments. Adapted from Tubbs et al., 2011.

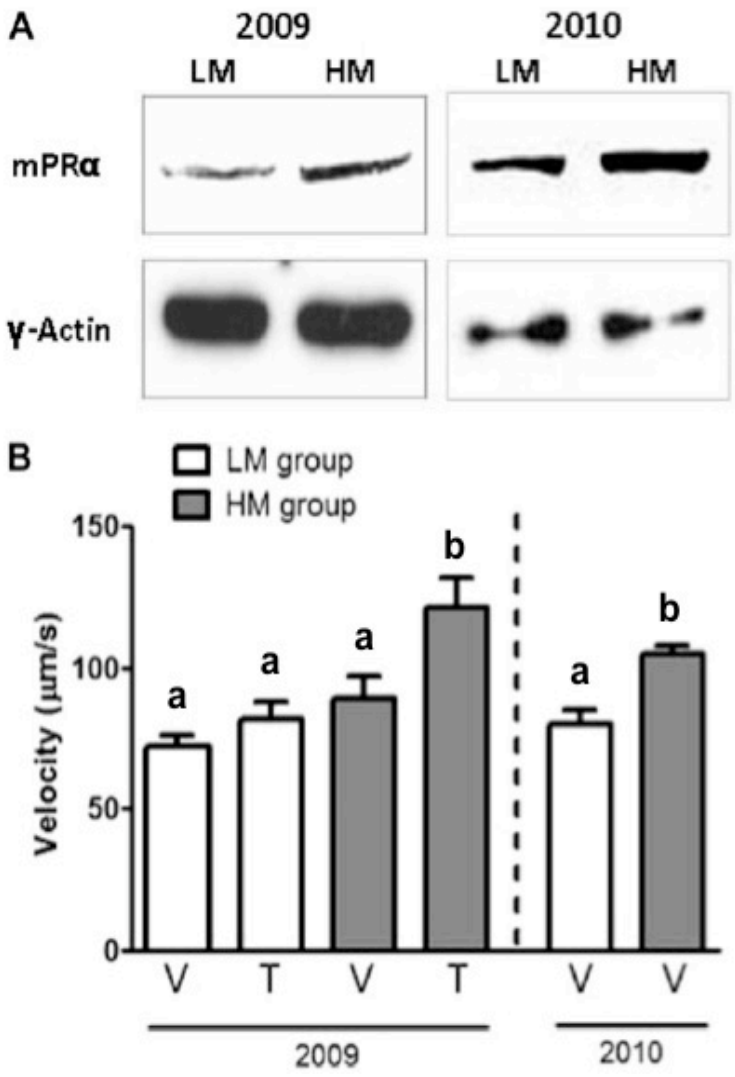
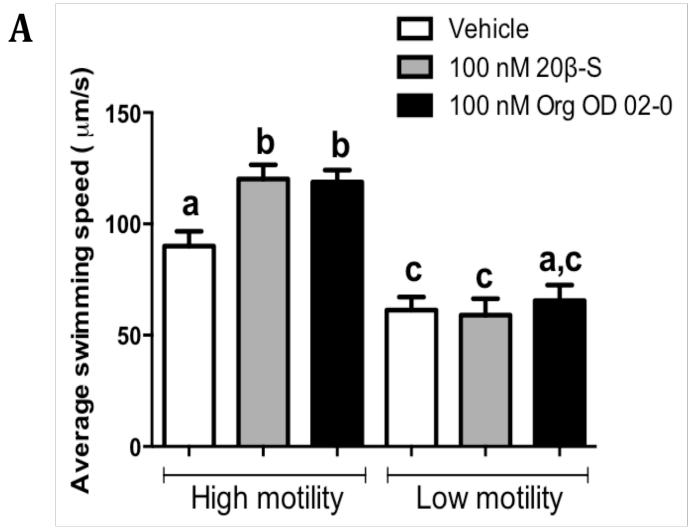
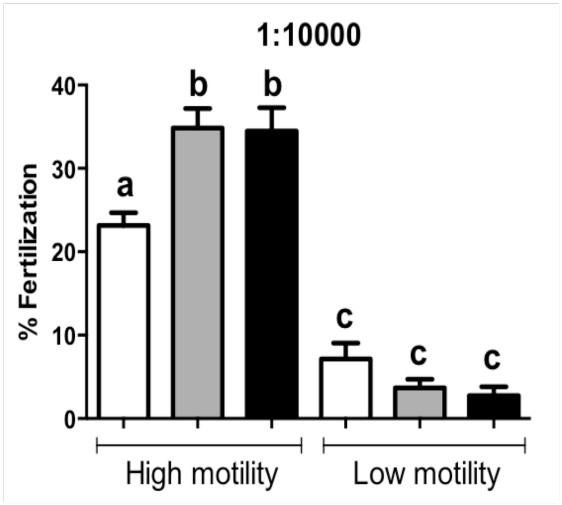


Fig. 4.3. Relationship between velocity and mPRα abundance of southern flounder sperm collected over two reproductive seasons. (A) Abundance of mPRα on sperm membranes from low motility (LM) and high motility (HM) sperm donors. Western blots show pooled sperm samples from 3 to 4 individuals for each group. Gel loading = 10 μg total protein. (B) Differences in sperm velocity of southern flounder sperm from high motility and low motility donors. Sperm were either treated with vehicle (V, 0.02% EtOH) or 20 nM 20β-S (T). All data represent mean ± SEM, n = 5 – 12. Shared letters denote no significant differences at $P<0.05$ level. Experiments were repeated four times and similar results were obtained each time., Significance was determined by one-way ANOVA and Tukey's post-test. Adapted from Tubbs et al., 2011.





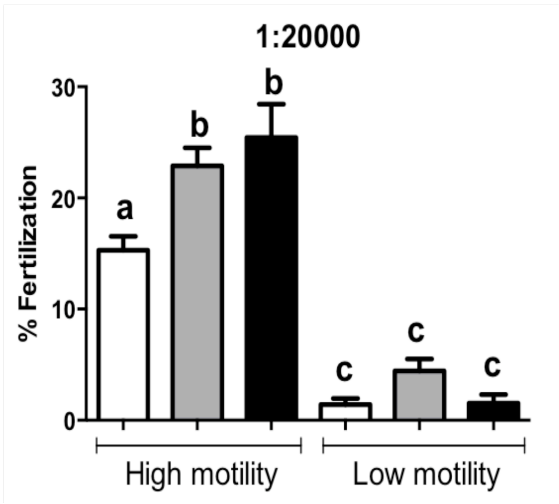


Fig. 4.4. Effects of 1 min treatment with 20β-S and the mPRα agonist (Org OD 02-0) on southern flounder sperm motility (A) and on fertilization success (B, C). A: Sperm motility assays were conducted with samples collected from freely milting males (high motility) and males that produced less milt (low motility). All data represent mean ± SEM, n = 7 – 9. Shared letters denote no significant differences at P<0.05 level. Experiments were repeated three times and similar results were obtained each time. B, C: Fertilization success was tested at two milt concentration: 1:10000 (B) and 1:20000 (C). All data represent mean ± SEM, n = 20 – 24. Shared

letters denote no significant differences at $P<0.05$ level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test. Spawning trials were repeated six times and similar results were obtained each time.

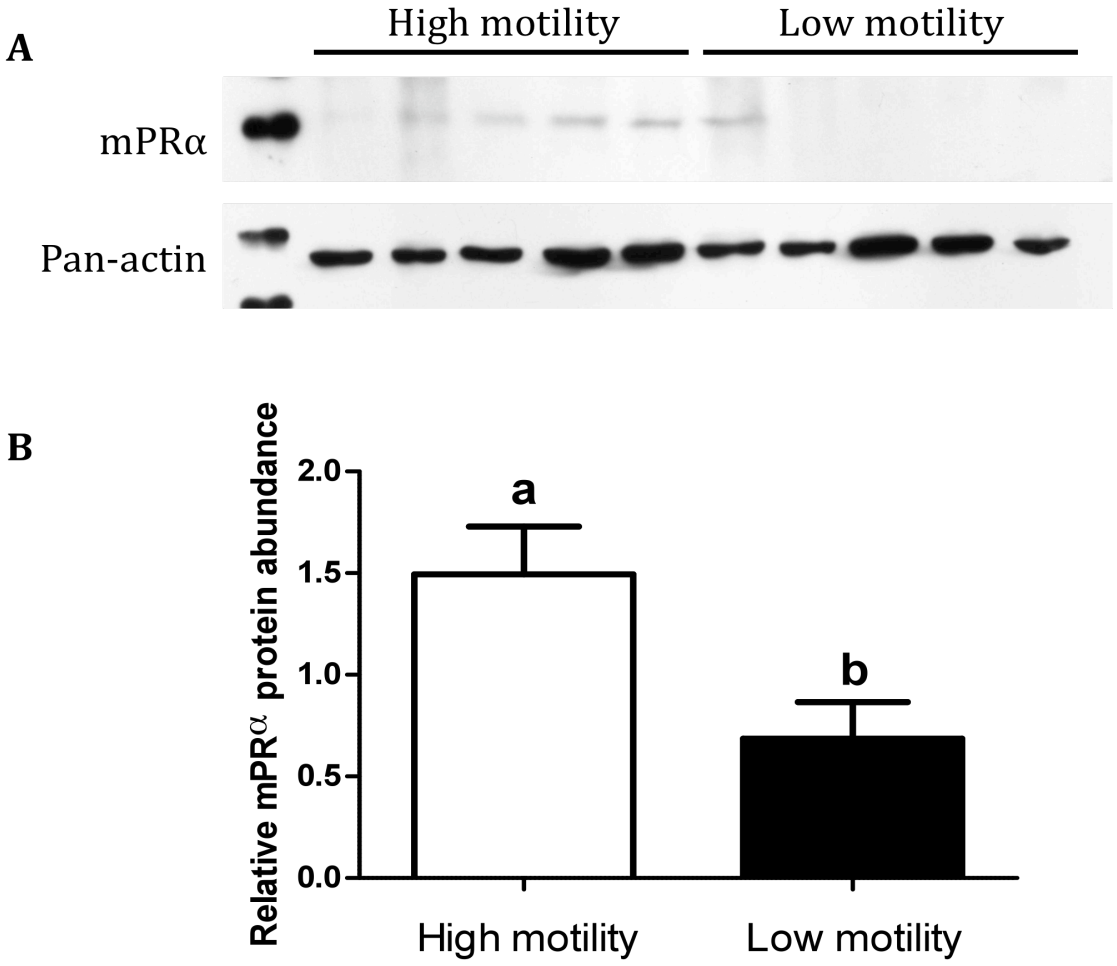


Fig. 4.5. Western blot analyses of mPRα protein concentrations on high and low motility flounder sperm plasma membranes. A representative Western blot (A) and the mean relative mPRα concentrations (B) are presented. All data represent mean ± SEM, $n = 6$. Shared letters denote no significant differences at $P<0.05$ level by Student's t test. The experiment was repeated three times and similar results were obtained each time.

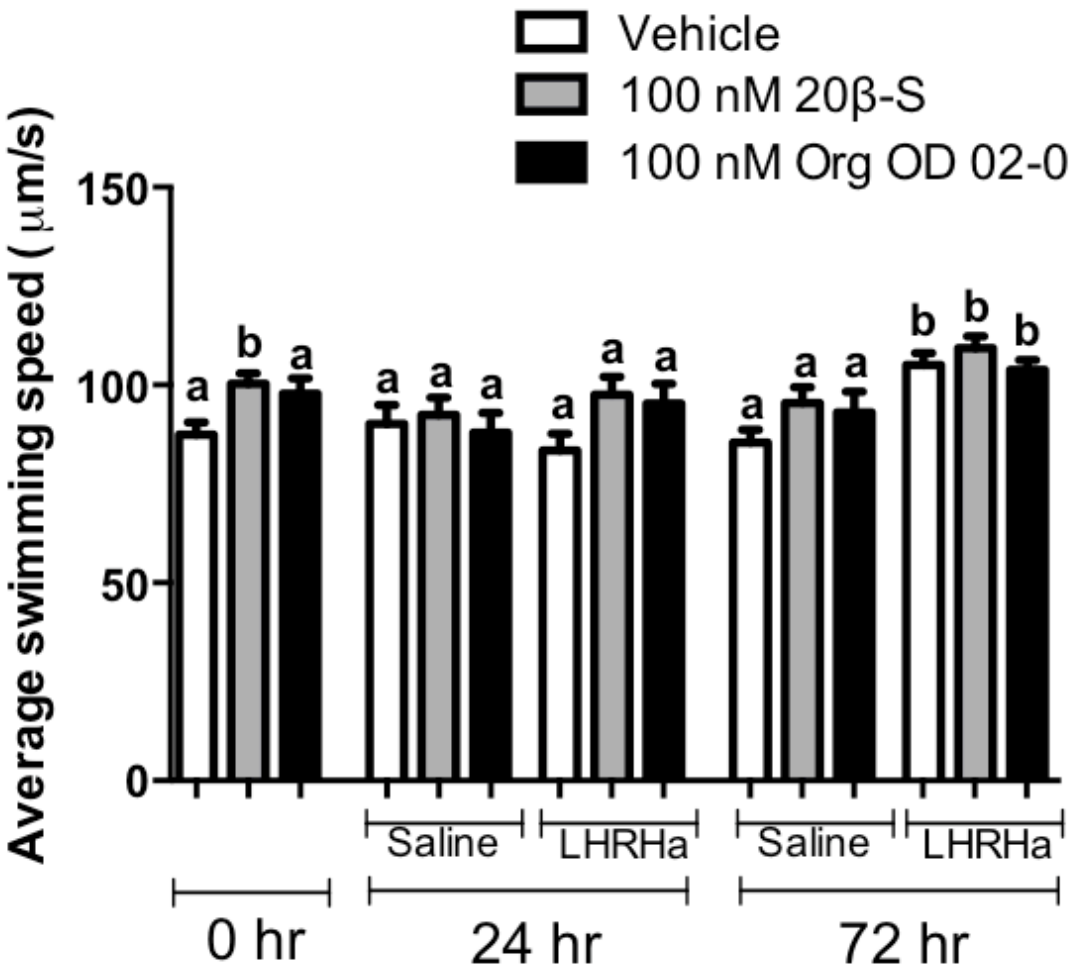


Fig. 4.6. Effects of a single LHRHa injection on flounder sperm motility. Males were injected with either saline or 100 μg/kg LHRHa and milt was collected at time points indicated. Sperm were treated with 100 nM 20β-S, 100 nM Org OD 02-0, or vehicle control. All data represent mean ± SEM, n = 20 – 45. Shared letters denote no significant differences at P<0.05 level from vehicle-treated controls at T = 0, calculated by one-way ANOVA and Dunnett's multiple comparison post-test. The experiment was repeated four times and similar results were obtained each time.

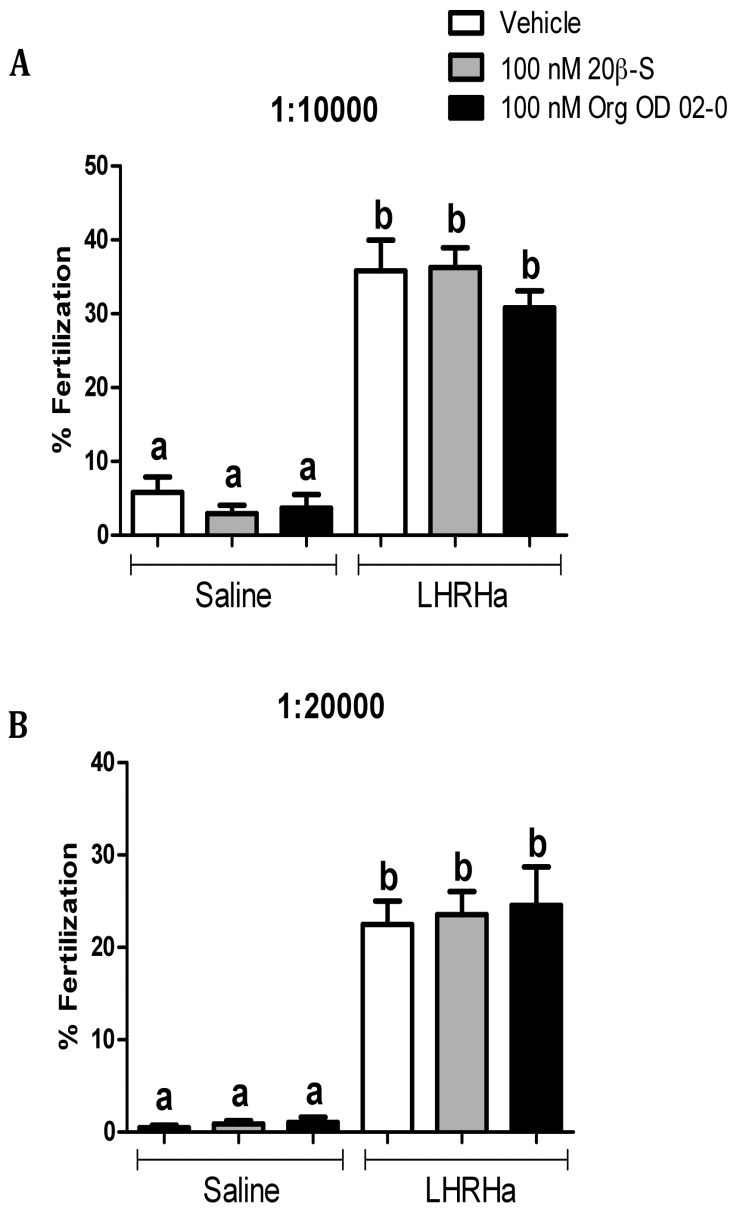


Fig. 4.7. Effects of a single LHRHa injection on flounder sperm fertilization success (A, B). Males were injected with either saline or 100 μg/kg LHRHa and milt was collected 72 h post-injection. Sperm was treated with either 100 nM 20β-S, 100 nM Org OD 02-0, or vehicle control before combined with strip-spawned eggs. Fertilization success was tested at two milt concentration: 1:10000 (A) and 1:20000 (B). All data represent mean ± SEM, n = 12. Shared letters denote no significant differences at P<0.05 level. Significance was determined by one-way ANOVA and Dunnett's multiple comparison post-test. The experiments were repeated three times and similar results were obtained each time.

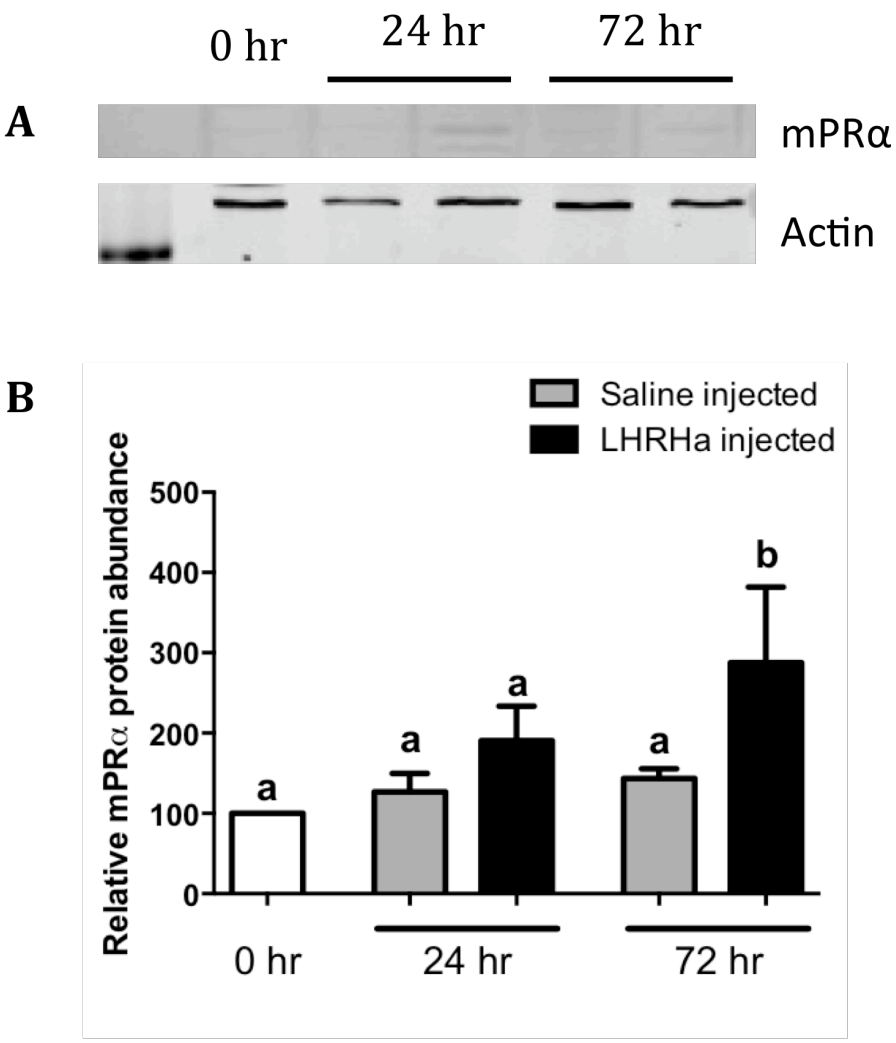


Fig. 4.8. Western blot analyses of mPRα protein concentrations on flounder sperm plasma membranes from fish injected with either LHRHa or vehicle. Mean relative concentrations (A) and a representative Western blot (B). All data represent mean ± SEM, n = 2 – 4. Shared letters denote no significant differences at P<0.05 level calculated by Student's t test. The experiment was repeated four times and similar results were obtained each time.

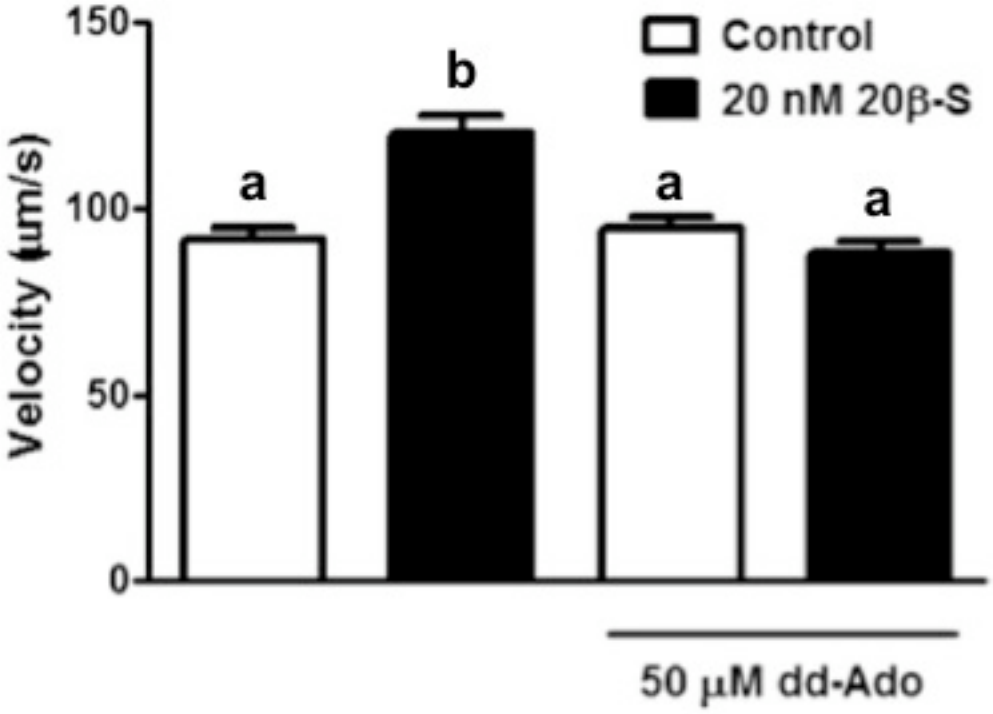


Fig. 4.9. Inhibition of progestin stimulation of southern flounder sperm hypermotility with an Acy inhibitor (dd-Ado). Data represent mean ± SEM, n = 9. Shared letters denote no significant differences at $P<0.05$ level from controls calculated by one-way ANOVA and Dunnett's multiple comparison post-test. The experiment was repeated three times and similar results were obtained each time. Adapted from Tubbs et al., 2011.

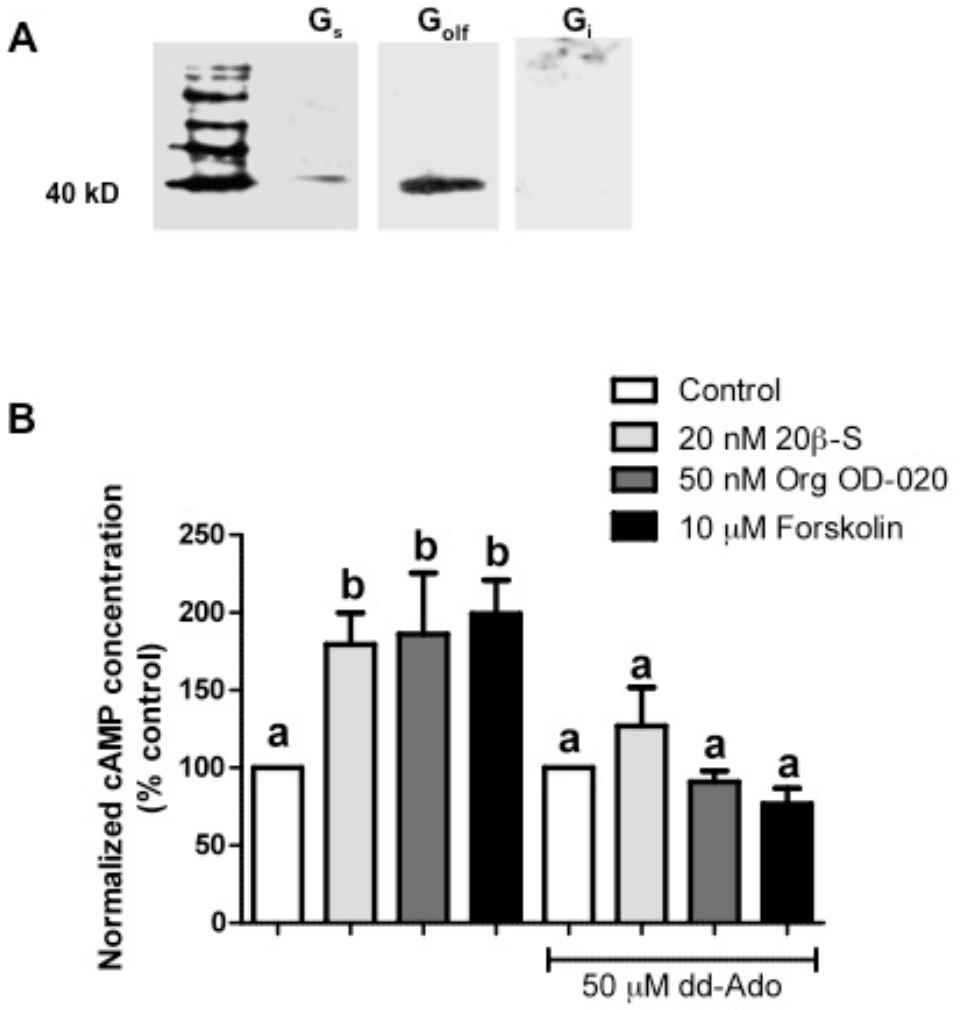


Fig. 4.10. Identification of signal transduction pathways in flounder sperm. A: Immunodetection of G proteins on flounder sperm plasma membranes by Western blot analysis. B: Effects of preincubation of flounder sperm plasma membranes with an inhibitor of Acy (50 μM dd-Ado) on cAMP production in response to treatment with either vehicle, 20 nM 20β-S, 50 nM Org OD 02-0, or 10 μM forskolin for 1 min. Concentrations of cAMP were normalized to the vehicle-treated control in each experiment to account for donor variability. All data represent mean ± SEM, n = 6 – 12.

Shared letters denote no significant differences at $P<0.05$ level from controls calculated by one-way ANOVA and Dunnett's multiple comparison post-test. The experiment was repeated four times and similar results were obtained each time.

Chapter 5: Seasonal hypoxia and simulated ocean acidification significantly impairs male Atlantic croaker reproductive potential

Abstract

Reproduction is highly sensitive to disruption by environmental stressors. Intense utilization of agricultural fertilizers has exacerbated seasonal aquatic hypoxia, resulting in prolonged and expanding dead zones. Over the past two hundred years, anthropogenic activity has also significantly altered the Earth biosphere by increasing atmospheric CO_2 to unprecedented levels, resulting in the acidification of the world oceans. Both issues are of particular interest to the long-term health of the Gulf of Mexico ecosystems. In this study, we show that chronic seasonal hypoxia in the northern Gulf of Mexico results in reduced gonadal and spermatogenesis in Atlantic croaker. Furthermore, sperm motility and the expression of the receptor that mediates progestin-induced sperm hypermotility, membrane progesterone alpha (mPRα) are also decreased. Experiments with croaker sperm activated in acidified activator solution also indicate that progestin-induced sperm hypermotility is abrogated at all three pH level tested: 7.6, 7.5, and 7.2. However, basal unstimulated sperm motility was not significantly affected until the pH reached 7.2. These results suggest that the mechanism mediating progestin-initiated sperm hypermotility in Atlantic croaker is more sensitive to environmental insult compared to the progestin-mediated mechanisms that control sperm motility. Taken together, this

study suggests that our rapidly changing oceans may have profound and detrimental effects on the reproduction processes of many marine teleosts.

Introduction

It is generally known that reproduction is especially sensitive to disruption by environmental stressors (Billard et al., 1981). Anthropogenic activity has had profound effects on the marine biosphere. Seasonal hypoxia has greatly increased in the northern Gulf of Mexico (GOM) over the past 30 years due to increased anthropogenic inputs of nutrients from the Mississippi River causing algal blooms (eutrophication) (Rabalais et al., 2007; Raymond et al., 2008). The long-term effects of recurring hypoxia exposure on marine ecosystems and valuable fisheries resources are unknown. Previously, Thomas and Rahman (2009) demonstrated in laboratory studies that chronic exposure of male Atlantic croaker to hypoxia (dissolved O_2 < 2 mg/L) resulted in reduced gonad size, disruption of the endocrine system, including decreased circulating teleost progestin hormone, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) levels, and a decrease in the abundance of the novel receptor on sperm plasma membranes that mediates the effects of 20β-S, membrane progestin receptor-alpha (mPRα, also known as Paqr7b). Furthermore, Thomas and Rahman (2009) also reported that exposure to chronic hypoxia also decreased croaker sperm motility, an important determinant of sperm quality (Fitzpatrick et al., 2008). These findings are consistent with the findings of Thomas et al. (2007) in a Florida estuary and more recently, from another study in the northern GOM (Thomas and

Rahman, 2011) demonstrating that spermatogenesis and its endocrine controls were severely disrupted in Atlantic croaker collected from hypoxic sites. However, sperm motility analysis on Atlantic croaker exposed to chronic seasonal hypoxia has not been performed in the field. Sperm motility analyses of samples collected from individuals trawled from areas affected by seasonal hypoxia would allow a direct correlation of the effects of seasonal hypoxia on an important functional aspect of male fertility as well as complement the previous studies cited.

Another largely unknown area of male gamete physiology of marine organisms is how sperm motility is affected by decreasing ocean pH levels. Since the onset of the Industrial Revolution, the pH levels of the world oceans has dropped by ~ 0.1 units and some estimates suggest that oceanic pH may continue to drop by ~ 0.4 units by the year 2100 (Caldeira and Wickett, 2003; Raven et al., 2005; Blackford and Gilbert, 2007; IPCC, 2007). Despite the belief that the oceans are chemically well-buffered, numerous reports suggest that ocean acidification threatens the viability of many species (Orr et al., 2005; Harley et al., 2006). Studies show that should the ocean pH levels drop by 0.4 pH units, there will significant adverse effects on physiological processes in a wide variety of species and ecosystems (Harley et al., 2006; Doney et al., 2009; Fabry et al., 2008).

Recent interest has turned to examining the effects of ocean acidification on the earliest life history stages of marine organisms, including external fertilization of eggs and larval development, which are highly susceptible to environmental changes. These stages are also key life stages for successful recruitment and continued survival of the species (Cowen et al., 2000; Raven et al., 2005). However, there is little data on how

lower oceanic pH levels will affect these processes (Fabry et al., 2008; Kurihara and Shirayama, 2004). Studies on sea urchins (*Heliocidaris erythrogramma*) have shown that CO_2-induced acidification by 0.4 pH units (~ pH 7.7) significantly reduces their sperm motility as well as the number of motile sperm, and has adverse effects on the developmental success of the embryos and larvae (Havenhand et al., 2008). In another study, Kurihara and Shirayama (2004) studied two other species of sea urchins, *Hemicentrotus pulcherrimus* and *Echinometra mathaei*, and reported significant declines in fertilization success at pH ~ 7.1. However, studies on the Pacific oyster (*Crassostrea gigas*) at pH levels lowered by ~ 0.35 pH units showed no significant effects on sperm swimming speed, percent motility, or fertilization kinetics (Havenhand and Schlegel, 2009). Furthermore, Kurihara et al. (2007, 2009) reported that there was no significant effect of ~ pH 7.4 treatments on fertilization success of the bivalves *Crassostrea gigas* and *Mytilus galloprovincialis* from Japan. However, it should be noted that these studies were conducted in varying sites around the world, with possibly varying seawater chemistry. It should also be noted that there is little or no data on how ocean acidification may affect the sperm physiology and reproductive success of vertebrates. Ocean acidification is further exacerbated in hypoxic dead zones as hypoxia is caused by the respiration of the sinking organic matter, which in turn also releases CO_2, thus also acidifying the subsurface water (Cai et al., 2011). The northern GOM is an area that experiences an annual seasonal dead zone that is both hypoxic as well as highly acidic (Wang et al., 2013). Thus, a study on the effects of ocean acidification on the sperm

physiology of the Atlantic croaker, a teleost species that is ubiquitous in the Gulf of Mexico, is particularly relevant.

In the present study, we tested the hypothesis that exposure to chronic seasonal hypoxia results in reduced gonad development, lower sperm production, lower expression of testicular mPRα, and reduced sperm motility. To the best of our knowledge, this is the first field study that attempted to directly link sperm motility with other reproductive parameters that are affected by seasonal hypoxia. Additionally, given that the GOM is an area that experiences an annual dead zone that is both hypoxic and acidic, the hypothesis that croaker sperm motility will be detrimentally affected by acidified media was also tested.

Materials and Methods

Chemicals

The Atlantic croaker progestin hormone, 17, 20β, 21-trihydroxy-4-pregnen-3-one (20β-S) was purchased from Steraloids (Newport, RI). All other chemicals and reagents were purchased from Sigma unless otherwise noted.

1. Field study on the effects of chronic environmental exposure to hypoxia on male reproductive functions

Wild Atlantic croaker were sampled by 10 – 20 min trawls at three normoxic sites (Ref: northern east of the Mississippi Delta around 180 km from the nearest dead zone site) and 12 hypoxic sites in the hypoxic dead zone area (east of the Mississippi Delta

along six transects C, D, F, H, J and S around 120 km apart, Fig. 5.1) during the reproductive season in October 2010. Apart from the reference sites, all sites along the other transects had experienced moderate to severe hypoxia two weeks prior to sampling. Milt (see below) and gonad samples were collected from at least six fish per site. Milt was used immediately for sperm motility analyses. One testis lobe was snap frozen for later determination of molecular and protein analysis, respectively. A piece of the second lobe was stored in 10% formalin solution for histological analyses of spermatogenesis. Fish carcasses were tagged and frozen for later determination of weight.

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin.

Milt collection

Milt was collected from fully mature male Atlantic croaker as described previously (Detweiler and Thomas, 1998). Briefly, the abdomen of the fish was wiped dry to prevent premature sperm activation by saltwater. Gentle pressure was applied to the abdomen and the milt was collected from the cloaca with a clean syringe and transferred to tubes that were kept on ice. Care was taken to prevent contamination of milt samples with urine, which would also cause premature activation of sperm.

Determination of % gonadal somatic index

The % gonadal somatic index (GSI) was determined by calculating the ratio of the mass of the gonad (the mass of the intact snap frozen testes lobe multiplied by 2) to the total body mass and multiplying the ratio by 100.

Preparation of sperm plasma membranes

Sperm membranes were isolated as previously described (Thomas et al., 1997) with minor modifications. Briefly, 3 – 5 mL of milt were diluted in 10 mL of ice-cold homogenization buffer [HAED; 25 mM HEPES, 10 mM NaCl, 10 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM EDTA (pH 7.6)] and centrifuged at 1000 × *g* to isolate sperm from seminal fluid. Sperm were resuspended in 10 mL HAED with protease inhibitors (Merck, Darmstadt, Germany). Sperm suspensions were forced through a 23.5 gauge needle twice and sonicated at medium power for 6 s on ice. Samples were then centrifuged at 500 × *g* for 10 min at 4 °C to remove the nuclear fraction. The supernatant was transferred to a clean tube and centrifuged at 17000 × *g* for 20 min at 4 °C to pellet the cell membrane fraction. Isolated sperm membranes were used immediately or stored at -80 °C. Protein concentrations of membrane preparations were determined using a Bradford protein assay (Bio-Rad, Hercules, CA).

Sperm motility analyses

Sperm motility experiments were performed as described previously (Detweiler and Thomas, 1998) with minor modifications. Briefly, croaker milt was diluted 200-fold and preincubated in physiological saline with 20 nM 20β-S or vehicle (EtOH, 1%) for 1 min at room temperature. A 2.5 µL aliquot of each sperm suspension was added to 25 µL activator solution on a microscope slide. A coverslip was placed on the slide and sperm motility was recorded for 1 min using a dark field microscope connected to a computer capable of recording high quality videos. Each experiment was recorded using a charge-coupled device camera (Cohu Electronics, San Diego, CA) and digital recording software (Pinnacle, Mountain View, CA). Each treatment was conducted in triplicate. Sperm

swimming velocity was determined using CellTrak motion analysis software (Motion Analysis Corp., Santa Rosa, CA).

Quantitative real-time PCR analyses

Total RNA from croaker testes was prepared as previously described (Tubbs et al., 2010). One-step qRT-PCR was performed using Brilliant II SYBR Green qRT-PCR Master Mix Kit, 1-step (Agilent Technologies, La Jolla, CA) following the manufacturer's instructions with 5 pmol of the following primers: *mPRα* sense 5'-GCTGCCTTCATCATCTTGGT-3', antisense 5'-CGCTGAAGGAGAGGTAGGTG-3'; 18S rRNA sense 5'-AGAAACGGCTACCACATCCA-3', antisense 5'-TCCCGAGATCCAACTACGAG-3'. Expression of *mPRα* relative to 18S rRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Western blot analyses

Approximately 10 µg of membrane protein was added to loading buffer [0.5 M Tris-HCl, 10% sodium dodecyl sulfate (SDS), 0.5% bromophenol blue, 10% glycerol] and resolved on 10% SDS-PAGE gels. After transfer to nitrocellulose membranes, membranes were blocked in a solution containing 5% nonfat milk, 0.1% Tween-20 in PBS [136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 10 mM KH_2PO_4 (pH 7.4)]. Membranes were rinsed with PBS and incubated overnight at 4 °C with primary antibodies directed against a 15-mer peptide sequence in the N-terminal region of croaker mPRα (YRQPDQSWRYYFLTL; 1:2500) in a blocking solution containing 5% nonfat milk and 0.1% Tween-20 in PBS. Membranes were rinsed with PBS and incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:5000; Abcam,

Cambridge, UK) in blocking solution containing 5% nonfat milk and 0.1% Tween-20 in PBS for 1 hr. Proteins were then visualized using SuperSignal West-Pico chemiluminescent substrate (Thermo Fisher Scientific Inc. Rockford, IL) and exposed to X-ray film (GE Healthcare, Buckinghamshire, UK). Membrane progestin receptor-α expression was normalized to pan-Actin (primary antibody incubation 1:10,000 in PBS and 0.1% Tween-20; secondary antibody incubation 1:5000 in blocking solution containing 5% nonfat milk and 0.1% Tween-20 in PBS) using ImageJ software (NIH) to obtain relative densitometries.

Histological analyses and estimation of sperm production

Testes preserved in 10% formalin were serially dehydrated in alcohol (70%, 80% and 90% ethanol). Prepared samples were sent off to Pacific Pathology, Inc (San Diego, CA) for processing. Sections were sliced to 5 μm thickness and stained with hematoxylin and eosin. Slides were examined using a Nikon Eclipse E600 microscope (Nikon, Japan) and images were captured by Cool-SNAP camera (Photometrics, Tucson, AZ). Gonads from three fish were selected from the Reference sites and three more were selected from Site S (the most impacted site based on GSI and sperm motility analyses) to represent the hypoxic sites. Three images were taken per slide. Images were analyzed using MetaVue software (Molecular Devices, Sunnyvale, CA). The ratio of the area occupied by mature sperm over total area of each image was estimated.

2. Laboratory study on the effects of decreasing pH on sperm motility

Animals

Adult Atlantic croaker were purchased in the fall from local bait shops. Fish were acclimated to the laboratory for two months in 35ppt salinity seawater before use in 12000 L recirculating tanks at 22 – 24 °C and a photoperiod of 11 hr light, 13 hr dark to promote and maintain gonadal development.

Effects of pH on sperm motility

For pH experiments, acidified activator solutions were prepared by decreasing the pH from 7.8 units to pH 7.6, 7.5, and 7.2 using HCl. Sperm motility experiments were performed and recorded as detailed above. The number of motile sperm per replicate was also counted to assess the effects of acidification on general sperm motility. As the size of the area analyzed is the same in all sperm motility analyses, the average number of total sperm per slide is assumed to be the approximately the same.

Statistical analyses

For all experiments, data are presented as means ± SEM. Statistical significance was determined using one-way ANOVA and Dunnett's multiple comparison post-tests on GraphPad Prism 5 Software (San Diego, CA).

Results

1. Field study on the effects of chronic environmental exposure to hypoxia on male reproductive functions

Effects of chronic seasonal hypoxia on the GSI of Atlantic croaker sperm

Fish sampled at the Reference sites had the highest mean % GSI (3.6 ± 0.4 %) indicating the presence of well-developed testes (Fig 5.2A, B). Fish with much smaller testes were found in sites that had experienced extreme hypoxia (shown in red in Fig 1) (Fig 5.2A, B). Site S was especially impacted (GSI 1.4 ± 0.2 %) followed by Sites J, H and F. Samples collected from Sites C and D indicated that fish at those sites were not as severely impacted as the other hypoxic sites.

Effects of chronic seasonal hypoxia on spermatogenesis in Atlantic croaker sperm

Hypoxic sites were represented by samples from Site S, as they were the most affected by hypoxia, based on the GSI data (Fig. 5.2A). Exposure to chronic seasonal hypoxia was correlated with significant reduction in spermatogenesis as shown by the reduced area occupied by mature sperm (sz) in the microscope images (Fig. 5.3A). The relative sperm production in testes from hypoxic areas (Site S) was 12.9 ± 1.7 %, compared to 37.3 ± 3.4 % in Reference sites (Fig. 5.3B). All images were taken at the same magnification.

Effects of chronic seasonal hypoxia on Atlantic croaker sperm motility

Basal sperm motility was significantly different between the Reference sites (122.8 ± 3.2 μm/s) and Site S (105.4 ± 3.2 μm/s) (Fig. 5.4). The 20β-S-induced sperm hypermotility was also significantly lower in Site S samples (112.1 ± 2.5 μm/s) compared to Reference sites (130.7 ± 2.9 μm/s) (Fig. 5.4). This is supported by the findings that GSIs (Fig. 5.2A) and sperm production (Fig. 5.3) in Site S was severely impacted by exposure to hypoxia. Sperm motility analyses of sperm from fish from Sites C and D indicate that sperm motilities were relatively unaffected in those sites. This is

corroborated by the finding that GSIs from both Sites C and D were also relatively unaffected (Fig. 5.2A). While basal sperm motility of samples from Site F was not significantly affected by hypoxia, the 20β-S-treated sperm displayed significantly lower sperm swimming velocities (106.9 ±4.3 μm/s) compared to 20β-S treated sperm from Reference sites (Fig. 5.4)

Effects of chronic season hypoxia on mPRα mRNA and protein expression in Atlantic croaker testes

Hypoxic sites were represented by samples from Site S, as they were the most affected by hypoxia, based on the GSI data (Fig. 5.2A). Relative mPRα mRNA expression in Atlantic croaker testes was significantly lower in hypoxia-exposed fish (0.018 ± 0.002) compared to fish sampled from Reference sites (0.025 ± 0.002) (Fig. 5.5A). Similarly, the relative abundance of mPRα receptor on the plasma membrane of croaker testes was significantly lower in hypoxia-exposed fish (1.2 ± 0.2) compared to fish from Reference sites (3.3 ±0.7) (Fig. 5.5B).

2. Laboratory Study on the effects of decreasing pH of activator on sperm motility

Atlantic croaker sperm motility was tested in the presence and absence of the endogenous progestin, 20β-S under different activator pH regimes. Sperm activated by regular activator solution (pH 7.8) responded to 20β-S by increasing the proportion of hypermotile sperm (characterized by higher swimming speeds, ~20 μm/s above basal swimming speeds) (Fig 5.6). At pH 7.6 (-0.2 pH units lower that that of regular activator solution), 20β-S-induced sperm hypermotility was eliminated (Fig 5.6A). However, there

were no significant differences in the number of motile sperm between treatments (Fig. 5.6B). At pH 7.5 (-0.3 pH units from regular activator solution), the same abrogation of progestin-stimulated sperm hypermotility was observed (Fig. 5.6C). While there were no significant differences in the number of motile sperm between treatments, there was a trend towards lower numbers of motile sperm under the more acidic activator regime (Fig. 5.6D). At pH 7.2 (-0.6 pH units from regular activator solution), we observed the same abrogation of 20β-S stimulation of sperm hypermotility (Fig. 5.6E). Additionally, there was also a significant decrease in the number of motile sperm when activated in pH 7.2 activator solution (Fig. 5.6F).

Discussion

The findings of this study are consistent with previous laboratory (Thomas and Rahman, 2009) and field studies (Thomas et al., 2007; Thomas and Rahman, 2011) using Atlantic croaker as a model species, in which it was shown that exposure to seasonal hypoxia had detrimental effects on Atlantic croaker male gonadal development, spermatogenesis, and the expression of mPRα message and protein. Additionally, we provide the first field evidence clearly showing that chronic seasonal hypoxia can have adverse effects on croaker sperm motility, confirming previous lab reports (Thomas and Rahman, 2009). *In situ* measurements of sperm motility at hypoxic field sites allow for direct correlations to be drawn regarding the effects of chronic hypoxia on croaker sperm physiology, a critical component of male fertility, and are thus ecologically important

data. It should be noted that only individuals from which milt could be expressed were selected for this hypoxia study. Milt could not be expressed from many of the fish collected from the hypoxic sites so that the results obtained with these relatively less affected fish may underestimate the severity of the reproductive impairment of the exposed population. This may explain the more muted response to hypoxia observed in the sperm motility assays compared to the GSI data, where only the most highly impacted site (Site S) showed a significant decrease in basal sperm motility. Two trawls were required to obtain enough milting fish from Site S, once again highlighting the severity of the impact of seasonal hypoxia at that site. The findings indicate that fish collected from Site S were consistently reproductively impaired at the levels of morphology of the testes (Fig. 5.2A), the significantly lower sperm production (Fig. 5.3), the impaired sperm motility (Fig. 5.4), and the expression of testicular mPRα (Fig. 5.5).

Hypoxia initiates a cascade of adaptive cellular responses including causing a shift towards anaerobic respiration, which involves increases in glycogen phosphorylase, aldolase and the increased production of stress-related proteins (Hochachka, 1986; Richards, 2009). This adaptation involves increases in mRNA transcription of genes for surviving anaerobic conditions and lipid metabolism (Richards, 2009; Martinez et al., 2006). Chronic hypoxia has been demonstrated to result in reduced intracellular ATP levels (Kim et al., 2006; Liu et al., 2006). Furthermore, mRNA translation and cell growth rates are also adversely impacted during hypoxia (Liu et al., 2006; Koumenis and Wouters, 2006). Hypoxia represses overall protein synthesis by inhibiting mRNA translation (Liu et al., 2006). These cellular and molecular responses are likely to be

mediated by hypoxia-inducible factor (HIF), a transcription factor that is activated during exposure to hypoxia in a wide variety of organisms (Semenza, 2011), including Atlantic croaker exposure to seasonal environmental hypoxia in the northern Gulf of Mexico (Thomas and Rahman, 2009b). All phases of spermatogenesis are controlled by gonadotropins (Schulz and Miura, 2002). Exposure to hypoxia has been shown to affect the croaker neuroendocrine system in controlled laboratory studies by decreasing the production of serotonin, which in turn decreases the expression of gonadotropin-releasing hormone (GnRH) mRNA in the hypothalamus resulting in reduced luteinizing hormone (LH) response to GnRH stimulation (Thomas et al., 2007). Previous work by Thomas et al (2007) and Thomas and Rahman (2012) clearly show that exposure to hypoxia also significantly lowers the levels of circulating androgens in Atlantic croaker. Thus, hypoxia can adversely affect spermatogenesis by abolishing the release of gonadotropins resulting in decreased androgen production, which are required for maintaining proper spermatogenesis. Furthermore, exposure of croaker to hypoxia has previously been shown to also lower the levels of circulating 20β-S, which is the maturation-inducing steroid (MIS) in this species (Thomas and Rahman, 2009). The reduced circulating MIS was associated with impaired oocyte development and sperm motility (Thomas and Rahman, 2009).

Additionally, as previously mentioned, hypoxic dead zones are also highly acidic due to the respiration of organic matter fluxes (Cai et al., 2011). Previous findings have shown that ocean pH levels under 7.7 significantly reduce the sperm flagellar motility of

reef invertebrates (Morita et al., 2010). Our results clearly show that at pH 7.6, the ability of croaker sperm to respond to the endogenous progestin and initiate sperm hypermotility was impaired. Similar results were observed with pH 7.5 and 7.2. However, the number of motile sperm was only significantly reduced at pH 7.2. These results suggest that the mechanism mediating sperm progestin-stimulated hypermotility is more sensitive to environmental insult than the mechanisms governing basal motility. Similar observations were made by Thomas and Doughty (2004) in that the progestin-stimulated sperm hypermotility mechanisms were more sensitive to xenobiotic chemical insult than the mechanisms mediating basal sperm motility. The sensitivity of the sperm of various organisms to ocean acidification varies significantly (Havenhand et al. 2008; Havenhand and Schlegel, 2009; Kurihara et al., 2007; 2009). The Atlantic croaker is able to tolerate moderate hypoxia and does not seem to display an aversion for hypoxic estuarine environments (Bell and Eggleston, 2005). Similarly, studies in bivalves (Havenhand and Schlegel, 2009; Kurihara et al. 2007; 2009), which generally inhabit dynamic estuarine environments that experience frequent hypoxia indicate a higher tolerance to ocean acidification. However, if progestin-stimulated sperm hypermotility is also a form of chemotaxis that aids in fertilization as suggested by Tubbs and Thomas (2009), results from the present study suggest that Atlantic croaker sperm may lose that ability at or around an oceanic pH of 7.6. Havenhand et al (2008) showed that statistically altered swimming speed and motility of sea urchin sperm resulted in 24.9 % decrease in fertilization success, which corresponded closely to their experimental findings (20.4 – 25.9 %) indicating that impaired sperm motility has detrimental effects on fertility. Thus,

decreases in pH may have profound effects on the fertilization success of Atlantic croaker spawns, which may have significant effects on recruitment and population dynamics. However, the present study utilized fish that were acclimated to current ocean pH levels and it is unknown if adaptations to decreased oceanic pH levels will attenuate some of the adverse responses observed in response to exposure to acidified activator solution.

In conclusion, our results clearly show that Atlantic croaker reproductive physiology is adversely affected by environmental insults. Chronic hypoxia has effects at the morphological, molecular and functional levels on testes to impair male croaker reproductive physiology. Additionally, the imminent further acidification of the oceans due to anthropogenic CO_2 emissions may have detrimental effects on croaker sperm motility and the ability of croaker sperm to initiate progestin-stimulated sperm hypermotility.

Figures

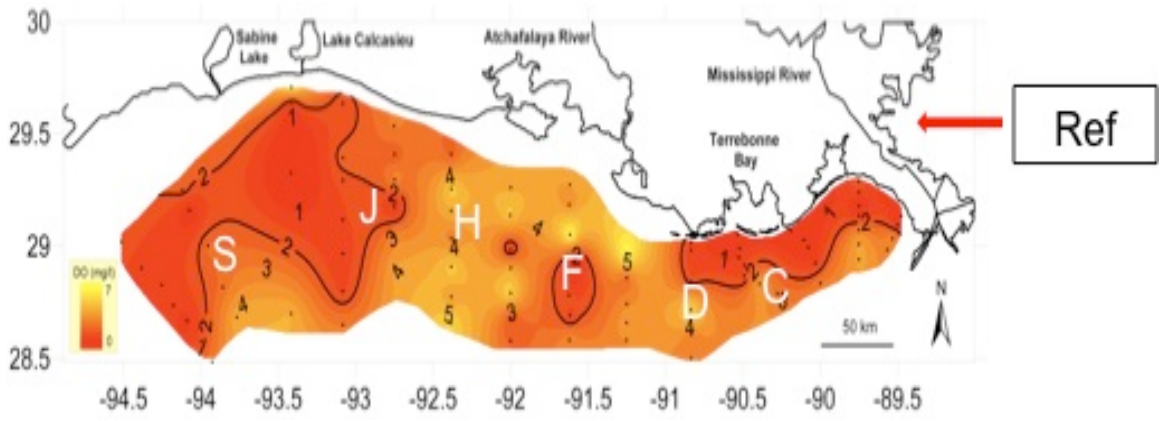


Fig. 5.1. Location of sampling sites in relation to hypoxic zone (summer 2010, map reproduced from N. Rabalais) in northern GOM. White letters indicate the sampling sites of the October, 2010 cruise.

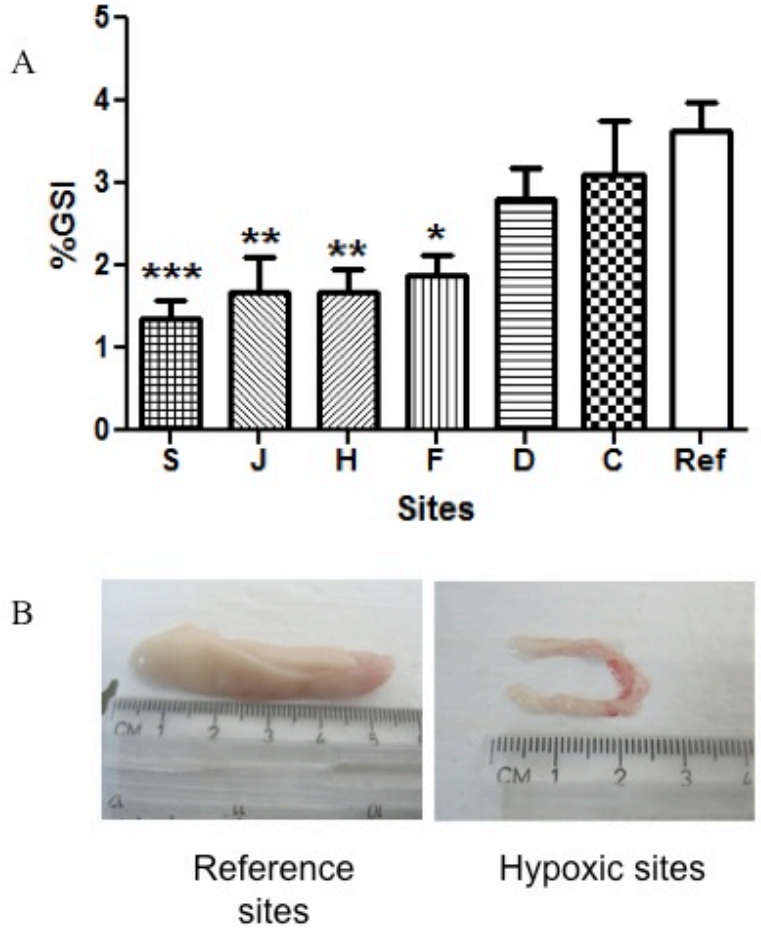


Fig. 5.2. Effects of chronic seasonal hypoxia on croaker testicular development (% GSI) of male croaker sampled from Reference (Ref) sites (pooled) and various hypoxic sites (A) labeled with letters corresponding to the locations marked on the map in Figure 1. Representative testes from Reference and hypoxic sites (B). All data represent mean ± SEM, $n = 6 - 11$. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$: significantly different from Reference by one-way ANOVA and Dunnett's multiple comparison post-test.

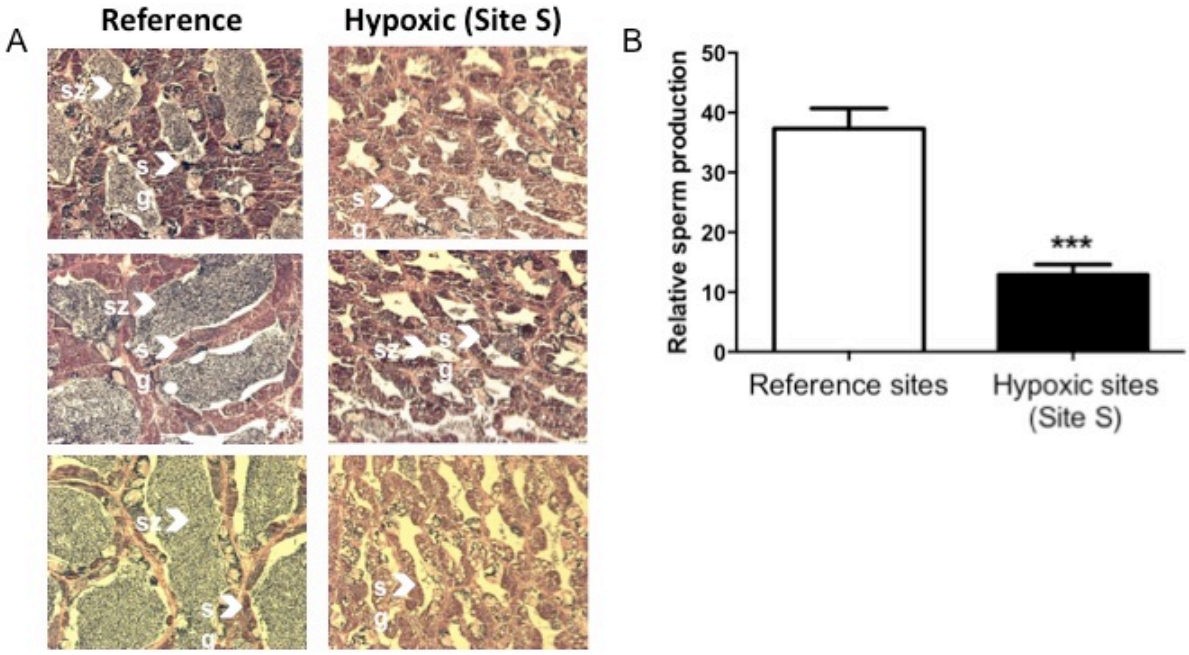


Fig. 5.3. Effects of chronic seasonal hypoxia on croaker spermatogenesis. Representative images captured of testicular histology from Reference and hypoxic sites (Site S) are shown (A). All images were taken at the same magnification. Mean relative sperm production from Reference and hypoxic sites (Site S) (B), sz mature spermatozoa, sg spermatogonia. All data represent mean ± SEM, $n = 8 – 9$. ***, $P < 0.001$: significantly different from Reference by Student's *t*-test.

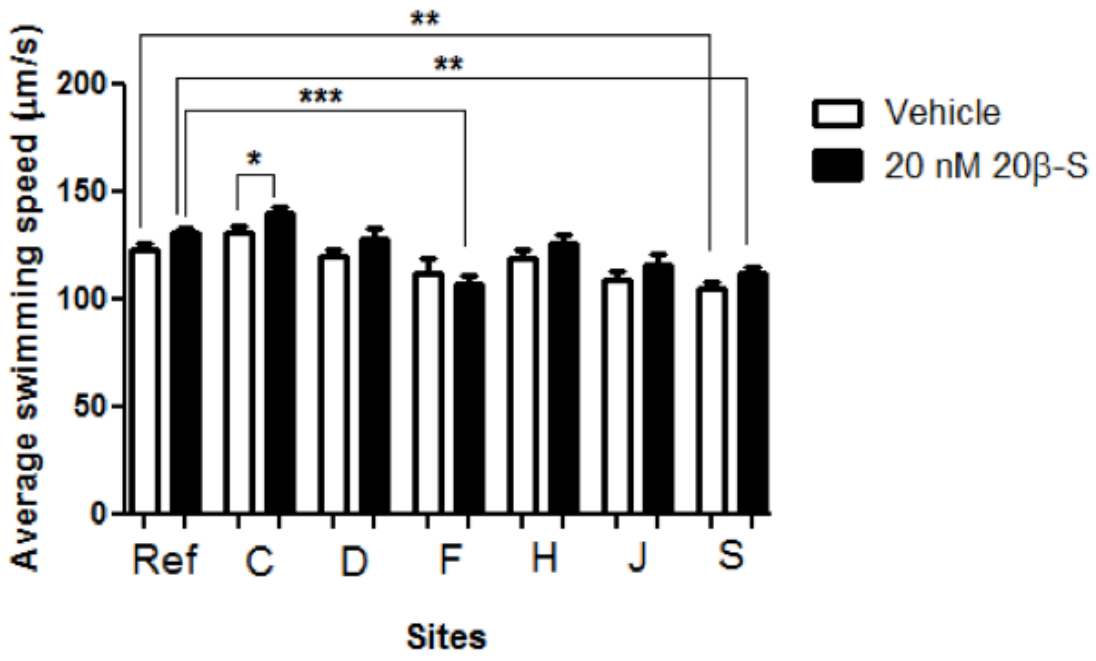


Fig. 5.4. Effects of chronic seasonal hypoxia on croaker sperm motility of croaker sampled from Reference (Ref) sites and various other hypoxic sites labeled with letters corresponding to the locations marked on the map in Figure 1. All data represent mean ± SEM, $n = 7 – 30$. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$: significantly different from Reference by one-way ANOVA and Dunnett's multiple comparison post-test.

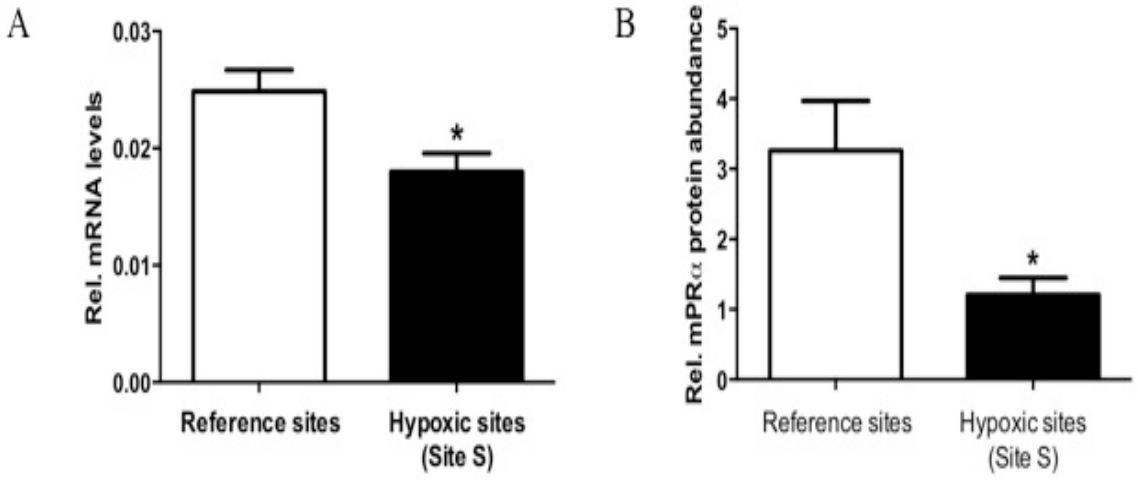


Fig. 5.5. Effects of chronic seasonal hypoxia on croaker testes mPRα mRNA and receptor expression. Mean relative croaker testes mPRα mRNA (A) and relative mPRα protein abundance (B) from Reference and hypoxic sites are shown. All data represent mean ± SEM, $n = 6 – 9$. *, $P < 0.05$: significantly different from Reference by Student's *t*-test.

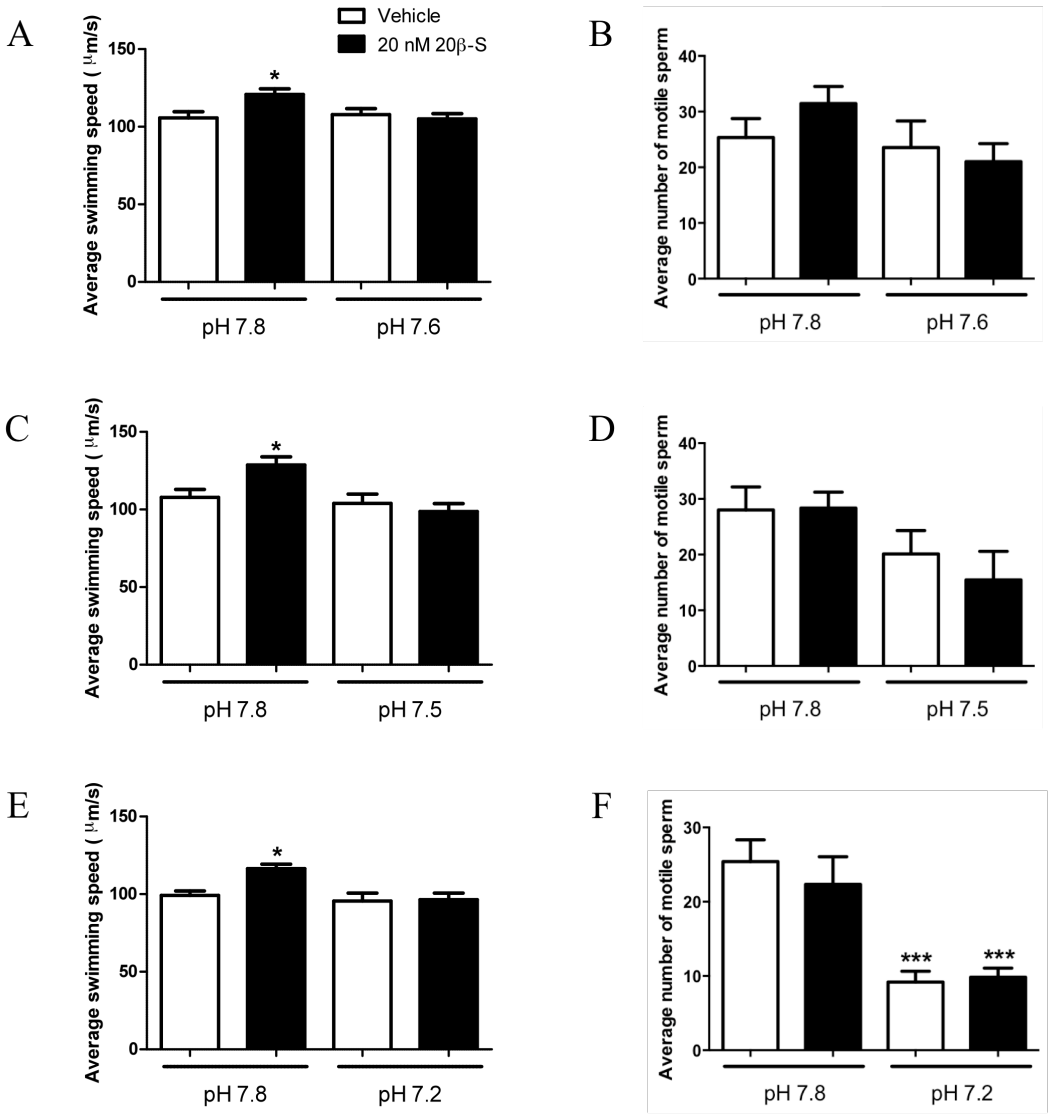


Fig. 5.6. Effects of acidification on croaker sperm motility. Average swimming speeds of sperm (A, C, E) and average motility (B, D, F) of croaker sperm are shown. All data represent mean ± SEM, $n = 9 – 12$. *, $P < 0.05$; ***, $P < 0.001$: significantly different from controls by one-way ANOVA and Dunnett's multiple comparison post-test. Experiments were repeated at least 3 times with each treatment run in triplicate.

Chapter 6: Summary and Conclusions

Results of the present study support the hypothesis that mPRα is the mediator of progestin-stimulated sperm hypermotility in both the Atlantic croaker and southern flounder, due to the fact that the mPRα selective agonist mimicked the stimulatory effects of the endogenous progestin in both species, 20β-S. The results of this study also indicate that progestins initiate two novel signaling pathways through the βγ-subunits of the stimulatory G_{olf} protein in croaker sperm: the Pi3k/Akt/Pde and ErbB2/Mapk pathways. The Pi3k/Akt/Pde pathway is particularly interesting as it suggests that intracellular cAMP levels are not solely under the control of the mPRα/$G_{\alpha\text{-}olf}$/Acy/cAMP pathway previously described (Tubbs and Thomas, 2009), but rather under dynamic modulation of intracellular signaling pathways activated by both the α- and βγ-subunits of the G_{olf} protein. The current findings also indicate that mPRα-mediated progestin-activated sperm hypermotility in croaker also involves the ErbB2/Mapk pathway, adding to the complexity of the signaling cascades involved in progestin-induced sperm hypermotility. Additional studies to examine how these different signaling cascades may converge will need to be performed to further understand how mPRα-mediated sperm hypermotility functions.

Additionally, the present study also indicates that mPRα mediated progestin-stimulated sperm hypermotility in southern flounder results in increased male fertility. *In vitro* exposure to both 20β-S and Org OD 02-0 were sufficient to significantly increase sperm hypermotility and fertilization success in this species, suggesting a possible avenue

of improvement for southern flounder captive spawns. Furthermore, the results of this study suggest that a single LHRHa injection of 100 μg/kg was able to restore high sperm motility and fertility to flounder 72 h post-injection, following an increase in expression of mPRα on the sperm plasma membranes. It was also shown that progestin signaling in southern flounder sperm is also dependent on Acy activity as inhibition of Acy using the specific inhibitor, dd-Ado, resulted in abolished progestin-initiated sperm hypermotility and cAMP increase. Further studies are required to verify that the signaling mechanisms first described in Atlantic croaker sperm are paralleled in southern flounder. However, the current findings strongly suggest that the progestin signaling pathways in teleost sperm are highly conserved.

This study also strongly suggests that male croaker reproductive physiology is detrimentally affected by exposure to chronic seasonal hypoxia. The field study results corroborate previous claims by Thomas and Rahman (2007; 2009; 2011). Of note is the sperm motility data that is the first conducted in the field. Sperm motility was shown to be severely impacted at some of the hypoxic sites sampled. Additionally, croaker sperm were also affected by activation in acidified activator solutions. The present results suggest that ocean acidification may have detrimental effects on both progestin-stimulated sperm hypermotility as well as general sperm motility. The progestin-stimulated sperm hypermotility mechanism appears to be more sensitive to environmental insult than the mechanism that governs general sperm motility. However, as the fish used in this study were not acclimated to extensive low pH regimes, it remains unclear if future adaptations may abrogate some of the severity of the low pH effects on croaker

sperm. Future studies on fish exposed to lower pH regimes will be required to make more conclusive claims. However, the present results indicate that sperm physiology is sensitive to environmental insults.

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